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PROCESSES OF CALCIFICATION IN THE CORALLINE
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PROCESSES OF CALCIFICATION
IN THE CORALLINE ALGAE

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ABSTRACT

Hawaiian reef coralline algae were used to study basic processes involved in mineralization. The coralline algae were chosen because there is little cellular differentiation, no subjection to hormonal influence, response to experimental changes in environmental conditions, and an extensive organic matrix. Investigations were conducted on coralline morphology, histochemistry and molecular structure, crystal composition and orientation, and on the role and structure of the organic matrix.

Coralline growth is optimal at approximately 17°C in diffuse light. Three growth forms are observed for corallines on cover slips: filamentous, fan-shaped, and disc- or fan-shaped with a fringe-like thallus periphery.

Histochemical tests indicate the presence of sulphated polysaccharides, protein, lipid and disulphide bonds in the thallus. A submarginal band of cells reacts most strongly with the dyes employed and also demonstrates metachromasia. Form birefringence is present in decalcified thalli, evidence of an highly ordered organic matrix.

The calcite polymorph of calcium carbonate is identified from X-ray diffraction and spectrometry as the crystal present in corallines. Crystal orientation in the thallus is determined by electron diffraction and electron microscopy. Three crystal locations and two crystal forms are observed. These suggest crystals are deposited first next to the cell, coalescing into larger units as the matrix and crystals move in the direction of growth. The presence
of periodicity similar to that found on the matrix fibres along some crystal edges, the smallest crystals next to the cell and the apparent engulfment of cover cells by crystals carried along the direction of growth tend to support this conclusion. Tabular crystals occur perpendicular to the cell with initial mineral precipitation noted at 150° intervals at the inner cell wall. Tabular crystals are also seen oriented parallel to the direction of growth but always of intermediate or large size and accompanied by cell edge crystals. Fibrous crystals are found intracellularly in random orientation in degenerating thallus areas. The fibrous (or spicule) mineral precipitation appears to be the result of a physical precipitation.

The organic matrix of coralline algae consists of numerous fine fibrils of random arrangement in sheets. Several periodicities are noted, apparently affected by fixation method and plane of sectioning. Calcium uptake is optimal at 29°C and most tightly bonded to the matrix at 17°C in cold decalcified matrix. Autoclaved decalcified matrix incorporates and binds calcium to a lesser degree than the non-autoclaved matrix. Non-autoclaved matrix takes up calcium against a concentration gradient, suggesting physiological processes, while autoclaved matrix reaches equilibrium with the medium only.

X-ray diffraction patterns, identical before incubation, differ in autoclaved and non-autoclaved matrices after post-decalcification incubation in a calcium-containing medium. The less numerous peaks in non-autoclaved matrix suggest that more regulation of calcium deposition takes place.

It is concluded that the coralline algae present a biological system capable of influencing calcification processes. A potentially
calcifiable matrix is demonstrated by development of metachromasia before mineralization occurs, strong basophilic properties of the tissue, fibre periodicity, and calcium incorporation. Matrix-crystal interaction is manifested by crystal orientation and location within an extensive organic matrix network. Physiological processes are suggested by greater calcium uptake and binding by cold decalcified matrix than by autoclaved matrix, slower uptake and weaker binding of calcium at temperatures below 10°C and above 29°C, and differences in X-ray diffraction patterns between decalcified autoclaved and cold decalcified matrices after incubation in a calcium-containing medium.
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INTRODUCTION

The purpose of this investigation was to elucidate basic factors essential to biological calcification. While a great deal of attention has been given to the mechanism of calcification in vertebrates and molluscs, other mineralizing systems have been neglected for the most part. The complexity of vertebrate systems, however, led to investigation of simpler organisms (for example, coccolithophorids). Recent research has focused upon the importance of the organic matrix, studying the ultrastructure and physiology which influences crystal deposition and growth (for a review, see Schraer, 1970). The present work was formulated to examine the cellular and molecular aspects of calcification independent of endocrinological interactions and cellular differentiation. Research was conducted on the heavily calcifying coralline algae to ascertain 1) the structure and role of the organic matrix and 2) whether physical and/or physiological processes were involved in crystal precipitation and arrangement.

Because very little work had been done on the coralline algae other than descriptions from light microscopy (Fritsch, 1959; Levin, 1962), background information on growth rates and cell structure and ultrastructure of some Hawaiian coralline algae necessarily preceded calcification studies. Crystal composition of the coralline algae had been previously determined (Baas-Becking and Galilher, 1931), and the role of environmental factors in cellular interactions was recognized as being important. However, orientation of crystals on
a cellular level was unknown. Previous work on corals (Wainwright, 1964) and molluscs (Wilbur and Watabe, 1963) using diffraction and electron microscopy produced excellent results. Therefore, those techniques and polarized light microscopy were employed to study crystal deposition and organization in the coralline algae.

The molecular structure and histochemical reactions of coralline thalli, but calcified and decalcified, were investigated to gain information on the processes involved in making an organic matrix calcifiable and on possible nucleation sites. It was hoped that by determining changes in the coralline thallus as it matured and became mineralized that such diverse factors as enzymatic complement, stereochemistry, and biochemical composition could be considered together in their relationship to calcification. Johnson (1960) found progressive changes occurring in the tendon of the turkey leg as it calcified, and it seemed worthwhile to investigate the possible changes taking place similarly as the coralline thallus deposited calcium carbonate. The association of sulphide bonds with calcifying areas (Douglas, Isenberg, Lavine and Spicer, 1967) and enzymes at locations of calcification (Simkiss, 1964; Enns, 1967) further supported the use of histochemical tests.

Physiological experiments with coralline thalli and the organic matrix were based on the premise that active uptake of calcium occurs. Calcium uptake and incorporation by the organic matrix were investigated, suggested by the work of Wilbur and Watabe (1963) with calcium carbonate precipitation on mollusc matrices. By varying conditions such as temperature, inactivation of the organic matrix, ion concentration and ion type, enzyme involvement in calcium
uptake could be considered in relation to enzyme activity and competition for reactive sites. Radioautography provided an opportunity to study the pathway of calcium and the locations of initial deposition. The models put forth by Glimcher (1953, 1960) for nucleation and crystal formation stimulated much of the work. And investigations on calcifying matrices (Schiffman, Martin and Miller, 1970; Wilbur and Watabe, 1967) provided information on which to design experiments to define parallels between coralline algae and other biological calcifying systems.
CHAPTER I.

Materials and Methods

Organisms

The coralline algae used in this investigation were collected from the reef behind the Waikiki Aquarium (at the eastern end of Mamala Bay), Oahu, Hawaii. Specimens were gathered throughout the year either as coralline encrusted rocks, heads of Porolithon gardineri, or small coralline thalli grown on cover slips. Collections of encrusted rocks and *P. gardineri* heads were made along the reef from the outer wave break to the natatorium circulation channel at depths of one to two meters. Special equipment was used for the growth of corallines on cover slips. Plastic holders (Figure 1) were fastened to a heavy concrete block (Figure 2) which was placed in about 1.5 meters of water at the inner edge of the wave zone, in an area of abundant *P. gardineri* growth. Exposure times ranged from two to eight weeks. Optimum coralline settling was found with 3-4 week exposures. This period provided sufficient time for the corallines but few competitive algae to grow. However, longer exposures were necessary during stormy periods; and shorter exposures sufficed when using plastic substrates, though the brown algae present increased, especially *Sargassum*. Coralline thalli from one cell to macroscopic size could be observed on one cover slip, with no correlation between settling and tides.

Laboratory Culture

The ocean collected coralline algae were transferred to plastic
Figure 1

Holder placed in the ocean to allow settling of coralline algae on cover slips. X 0.5.

Figure 2

Concrete block with holders attached in the ocean. X 0.08.
laboratory aquaria kept well aerated, at 22°-23° C, and in diffuse light. Diatom growth presented a problem, but the addition of 1.5 mg/liter of GeO₂ (Lewin, 1966) reduced the diatoms without visually affecting the corallines. Most contamination could be removed from the cover slips by gently rubbing with the fingers, because the coralline algae adhered more strongly than the other algae. White (dead) coralline thalli also were removed in this manner, while the pink (viable) thalli remained. Sea water obtained from the Waikiki Aquarium pump was used unless there was a special need for artificial sea water. Artificial sea water was prepared according to the formulae of Rice, 1954 (enriched sea water), McLaughlin and Zahl, 1959 (M-8 medium), and Gold, 1964 (Gold's medium). Of the artificial sea waters tested, Gold's medium gave best results for maintaining live coralline algae.

**Light Microscopy**

For light microscope observation of the living coralline thalli, the cover slips with coralline growth were placed in small plastic chambers (Figure 3) of sea water. Low light intensities were used to avoid damaging the thalli, and photomicrographs required exposures of five seconds on Kodak Panatomic X film (ASA 32) with an Asahi Pentax Spotmatic camera (f 1.4). Photomicrographs of the fixed and stained corallines was done with a Zeiss Universal microscope with an automatic camera attachment, using Agfachrome film (ASA 50). Some photomicrography was done using Kodachrome II and Ektachrome film, but the results tended to favor the red or the blue end of the color spectrum, respectively.
Figure 3.

Water-tight culture chamber for maintenance of cover slip grown coralline algae in medium while viewing through a microscope or for control of incubating conditions.
Geological thin sections were prepared of dried *Porolithon gardineri* for birefringence studies. Fragments of *P. gardineri* branches were mounted on glass slides with balsam, orienting the desired section parallel to the slide. The algal fragment was next sawed (if thick) and/or sanded parallel to the slide to the predetermined depth desired for observation. The balsam was then melted, the algal piece turned over, and the flattened side pressed tightly against the glass slide. After the balsam hardened, the specimen was carefully sanded to the very thin section necessary — first on a rotary grinder and finally with pumice on frosted glass. Thin sections and cover slip algae were examined for birefringence with polarizing lenses in a Zeiss Universal microscope, used through the courtesy of Dr. L. I. Rehbun. Photomicrographs were taken with the automatic camera on Kodak Plus-X film and developed in Diafine developer.

**Electron Microscopy**

Tissue used for electron microscopy included 1-2 mm fragments of *Porolithon gardineri* and encrusting corallines, pieces of coralline thalli scraped from the sides of aquaria and from plastic pieces, and coralline growth on cover slips. The cover slips were manipulated through the series of solutions in cover slip staining dishes, and the tiny fragments were placed in small vials.

Optimal fixation was in aqueous 0.5% BaMnO₄ at 4°C for 20 minutes (Mollenhauer and Zebrun, 1960) or 2.5% gluteraldehyde paraformal in 0.1 M cacodylate buffer at 4°C for 20 minutes (Barka and Anderson, 1967). Because tissue fixed in the same
manner showed wide variability in section quality, it should be pointed out that some of the other fixation methods may have value, in spite of initially poor results. Other fixations employed are given in Table 1. Overnight rinsing followed all fixations, with several changes of the buffer used originally to make up the fixative.

Dehydration of the tissue was in 50%, 75%, 95% ethanol and three changes of absolute ethanol. This was followed by three changes of propylene oxide. Each step required approximately 15 minutes.

Tissue embedding was initiated by adding 1:1 propylene oxide and embedding mixture to the specimens for a minimum of one hour. Then 100% plastic was added and left overnight at room temperature, followed by fresh plastic for one hour or more. Final embedding of the tissue pieces occurred in the tip of polyethylene Beem capsules or flat embedded in the capsule cap. The specimen was carefully lifted from the infiltration vial on the tip of a pointed wooden stick and the excess plastic allowed to drain off. The tissue was placed in a drop of fresh plastic in the final embedding container and positioned after the plastic had thickened (2-4 hours). The capsule was then filled with plastic and polymerized at least 48 hours at 60°C. Cover slips were carefully drained of plastic and placed thalli side down on top of plastic tissue culture "blisters" (Blister Microscope Slides) filled with fresh plastic. Care was taken to angle the cover slip down, and, as a further precaution against bubbles, the embedding holders were kept under vacuum overnight. After polymerization for 48 hours at 60°C, pieces of cover glass could be chipped from the underlying plastic by gently lifting
Table 1

Methods for Fixation of Coralline Algae for Electron Microscopy

2.5% gluteraldehyde in phosphate buffer (Sabatini, Bensch, and Barnett, 1962) with postfixation in 1% OsO₄ (Palade, 1952)

0.6% KMnO₄ in veronal-acetate buffer (Luft, 1956)

1.2% cold aqueous KMnO₄ with postfixation in Palade's OsO₄ (McAlear, 1962)

OsO₄ vapor for ten minutes followed by 2.5% gluteraldehyde in phosphate buffer

2.5% gluteraldehyde in phosphate buffer followed by aqueous 1.2% KMnO₄

6.5% gluteraldehyde in phosphate buffer followed by aqueous 1.2% KMnO₄

6.5% gluteraldehyde in phosphate buffer followed by Palade's 1% OsO₄

2% OsO₄ in veronal-acetate buffer with 1.5% sucrose added (Caulfield, 1957)

1% OsO₄ in veronal-acetate buffer followed by 0.5% uranyl acetate in veronal-acetate buffer (Kellenberger et al., 1958)

Luft's KMnO₄ buffered with veronal-acetate and made isotonic to sea water with NaCl
one edge of the glass. If dry ice was touched to the cover slip first, the tissue separated more easily from the cover slip and remained embedded in the plastic. The plastic mixture which gave the best results was Epon 1:1 (Luft, 1961). Other plastic mixes used were Epon 1:3 and 1:5 (Luft, 1961), Epon/Araldite for pollen grains (Mollenhauer, 1964), Araldite (Glauert and Glauert, 1958), Maraglass, and New Epon (McGee and deBruijn, 1964).

Tissue embedded from cover slips had to be sectioned parallel to the surface or re-embedded by placing a small piece of plastic and tissue in a container of fresh plastic. The tissue was sectioned directly in the capsule tip or from the flat embedded specimens. In some instances, the tissue was re-positioned by cutting out a small block of plastic containing the tissue and remounting it on a plastic block with epoxy resin. It was necessary to trim the blocks to an extremely small trapezoidal face in order to section the calcified tissue. Sectioning was done at the slowest setting on automatic ultramicrotomes, producing sections of around 600-800Å. Both glass and diamond knives were used. The best sections were achieved with a Reichert OM-U2 ultramicrotome, but a Servall MT-1 and LKB 4800A were also used.

The sections were picked up on copper grids with collodion films (0.5% collodion in amyl acetate) coated with carbon. Formvar and carbon films were also used, but collodion gave the most consistent results. The small sections necessitated the use of film support.

Staining was achieved with lead citrate (Reynolds, 1963) and/or barium permanganate (Pease, 1964). Other stains occasionally employed were lead hydroxide (Karnovsky, 1961) and 5% uranyl acetate in 50%
methanol.

Initial electron microscopy was done with a Philips 75 electron microscope using Plus-X film for image recording. Most of the microscopy, however, was performed on an Hitachi HU-11A at 50Kv. Electron micrographs were taken on Kodak high contrast projector slide plates (3½" x 4"). Occasional microscopy was done on a Philips 300.

Shadow casting was done on some grids. Approximately 3.75 cm of 80% Pt - 20% Pd, 8 mil wire was evaporated onto the grids at an angle of 30°.

Staining for acid phosphatase location, according to the method of Barka and Anderson (1967) produced excellent technical results.

Decalcification

Different decalcification techniques were used for specific purposes. The most widely used decalcification method was neutral 4.13% EDTA (disodium, ethylenediamine-tetracetic acid) at 4° C (Warshawsky and Moore, 1965). The decalcification was slow, taking up to two months for pulverized Porolithon gardineri, but the method purportedly leaves active enzymes. Other methods used were 10% EDTA, 10% HCl, 1% chromic-acetic acid plus urea, 5% glacial acetic acid, and 7.5% glacial acetic acid combined with an ultrasonic generator (Thorpe, Bellomy and Sellers, 1963). All were extremely harsh on tissues. Simultaneous fixation and decalcification was tried using 10% formalin and 5% disodium EDTA (Manning and Butler, 1965) with results inferior to the cold neutral EDTA.

Grids with sections for electron microscopy were floated on decalcifying solutions (1% phosphotungstic acid and 2.5% EDTA) for
times ranging from ten minutes to one hour (Watabe, 1963). However, decalcification was slight if occurring at all.

Radioisotopic Tracers

Live coralline thalli on cover slips and decalcified geological thin sections were labelled with radiocalcium-45 and radiosulphur-35. The cover slips were suspended in Gold’s artificial sea water with radiocalcium substituted for the required calcium or in natural sea water with either radiocalcium or radiosulphur added. The cover slips were removed periodically for pulse labelling, incubation times ranging from 0.5 to 48 hours. After incubation, the cover slips were immediately placed in 2.5% gluteraldehyde in phosphate buffer at 12°C for three hours or at room temperature for 15 minutes. After overnight rinsing in Sorenson's phosphate buffer (Umbreit, Burris and Stauffer, 1957), the cover slips were transferred to distilled water. The radiosulphur-35 incubated cover slips were dipped in radioautographic subbing gelatin (0.5% gelatin, 0.05% ferrous ammonium sulphate in double distilled water) and allowed to dry at room temperature. The dry preparations were dipped in Kodak NTA liquid emulsion for two minutes, dried at room temperature in front of a fan, and the dipping and drying repeated. The slides with emulsion were stored in a light proof box at 10°C for exposure to the radionuclide. The radiocalcium-45 incubated cover slips were given identical treatment, except that no radioautographic subbing gelatin was used and Kodak NTA liquid emulsion was applied.

The radiocalcium-45 labelled corallines (43.9 μCi/liter) were exposed for two weeks. The radiosulphur-35 labelled corallines were
exposed for periods of two weeks (48 μCi/liter) and five weeks (16 μCi/liter). The films were developed in Kodak D-19 developer for about eight minutes, rinsed in tap water, and fixed in Kodak rapid fixer for five minutes. After rinsing for an hour in running tap water, the cover slips were stained in haematoxylin and eosin and mounted with Permount.

The decalcified geological thin sections were suspended in calcium-free Gold's medium with 10 μCi/liter of radio-calcium added. Samples were removed periodically and monitored with a Geiger-Müller portable monitor.

Uptake of calcium by *Porolithon gardineri* was followed by incubating 1-2 mm pieces of semi-decalcified matrix in radiocalcium-45. After a month or more in cold, neutral EDTA, the algal pieces were rinsed several times in calcium-free Gold's artificial sea water. A portion of the matrix was autoclaved at 20 p.s.i. for 15 minutes at 250°C, then rinsed in calcium-free Gold's medium. After concentration by centrifugation to a thick slurry, the matrix was added to flasks containing calcium-free Gold's artificial sea water enriched with approximately 10 μCi/liter of 45CaCl2. Sample aliquots were pipetted onto millipore filters placed on a fretted glass filter. Vacuum was applied to dry the sample. The matrix on the filter was rinsed with 1 ml of neutral Tris buffer at 0°C, followed by an additional 0.5 ml after the first rinse was filtered through. The millipore filter was placed on a planchette and dried at room temperature. If the filter curled, acetone was added to the planchette to dissolve the filter and flatten the sample. After thorough drying,
the samples were counted using a Nuclear Chicago gas flow counter.

**Diffraction**

Electron diffraction of powdered *Porolithon gardineri*, encrusting coralline, *Halimeda discoidea*, dried thalli of *Padina japonica* and standards of aragonite and calcite crystals was done with an Hitachi HU-11A electron microscope at 100Kv. Adhesive made of household cement dissolved in acetone was dropped on top of powder placed on a copper grid and allowed to evaporate. Diffraction was extremely difficult, because the Hitachi used was not set up for diffraction, but powder rings and crystal orientation were obtained. Focus was centered on the edge of a crystal, the field limiting aperture put in and closed to its lower limit, magnification reduced to zero and the beam used at cross-over. A diffraction "spot" was lowered over the intense center light, and electronmicrographs were taken for exposures of about 60 seconds.

X-ray diffraction was done on the same specimens as were used for electron diffraction except for the absence of crystal standards. The semi-decalcified matrix of *Porolithon gardineri* was filtered and dried and diffracted, also. The powders were evenly distributed on glass slides and held in place with adhesive in the case of the algal powders, or packed into powder holders placed on glass slides in the case of matrix diffraction. Diffraction was done on a Norelco X-ray diffractometer using a copper tube and voltage of 35 kV and 20 ma. The samples were scanned from 3° to 80° 2θ, using a Geiger-Müller gas flow detector for recording.

A Norelco X-ray spectrometer was utilized for composition analysis.
of *Porolithon gardineri* and encrusting coralline powders. A tungsten tube and ADP crystal were used to scan from 10° to 145° after powder preparation identical to that for X-ray diffraction. Standards of the tungsten tube and glass slide were used for comparison.
CHAPTER II
Morphology and Growth

Introduction

In order to understand coralline synthesis and morphology, it was thought appropriate to present a brief introduction to coralline growth, form and general ultrastructure. These will serve to illustrate growth patterns and their change by environmental conditions, cell type, and cellular organelles that may be important to an understanding of calcification.

The coralline algae are heavily calcifying, crustose or articulated red algae (Figure 4). The phylogenetic classification is (Dawson, 1966):

Division: Rhodophyta
Subclass: Florideae
Order: Cryptonemiales
Family: Corallinaceae

Subfamily: Melobesioidae (crustose and nodular)
Genera: Lithophyllum, Lithothamnion,
        Melobesia, Porolithon

Subfamily: Corallinoideae (crustose base with erect articulated parts)
Genera: Corallina, Jania

In this investigation, no attempt was made to determine genus or species of the very small, monostrromatic thalli. Some characteristics used to distinguish the crustose corallines from other algae were pit connections (Figure 5), granular appearance of older cells
Figure 4

Coralline algae commonly found in Hawaiian reef waters, *Porolithon gardineri* (above) and nodular encrusting corallines on rocks (below). Both X 0.67.
(Figure 5), and rectangular shape of cells (Figure 5). Megacells (heterocysts or trichocytes) (Figure 5) and lateral cell fusions (Figure 6) also are characteristic of coralline algae but are not always present. Identification of Porolithon gardineri was made by the presence of horizontal groups of megacells; and numerous lateral cell fusions in regular patterns indicated Melobesia. Jania, an articulated coralline, was found only a few times on cover slips, but this scarcity may have been due to the short growing times preventing the development of the identifying articulated branches. Unless identification was positive, the thalli are referred to as "coralline" algae.

The corallines appear reddish to purplish pink due to the phycoerythrin and phycocyanin pigments, bleaching to very light pink or white when dead. Reproductive spores are produced in conceptacles, the polystromatic, fertile areas on crustose thalli (Figure 6). After release and settling, the spores grow out laterally to form the thalli. The only spores that I could identify as coralline were cruciate tetraspores (Figure 7), and in most small thalli, a cell with the conspicuous "cross" could be found central to growth.

Fluorescence was observed when coralline algae were illuminated with ultraviolet light. Heads of Porolithon gardineri and rocks encrusted with corallines fluoresced a bright orange color due to the xanthophylls. Corallines grown on cover slips fluoresced yellow on the margins and a very deep red in the cell interior (Figure 8). Decalcified thalli also fluoresced yellow on the cell edges, suggesting that incident light produced the emission and not calcium
Figure 5
Photomicrograph of a monostromatic coralline alga growing on a cover slip showing cell walls, cw; cover cells, cc; megacells, mc; lateral fusions, lf; the granular cell appearance and rectangular cell shape. The thallus periphery, p, is indicated. X 1,000

Figure 6
Photomicrograph of a coralline tentatively identified as Melobesia membranacea growing on a piece of glass showing probably conceptable regions, co. No megacells are present. X 200.
Figure 7

Cruciate tetraspore photographs on a cover slip. The identifying "cross" can be found centrally located in most small coralline thalli. X 800.

Figure 8

Photomicrograph of a coralline alga on a cover slip using ultraviolet light. X 300.
carbonate. The deep red color was due to chlorophylls and phyco-bilins in the chromatoplasts.

Three main morphological types of coralline growth were commonly found on the cover slips: 1) filamentous without megacells (Figure 9); 2) disc- or fan-shaped with a fringe-like periphery with megacells (Figure 10); and 3) branching in fan shapes with megacells (Figure 11). The overall impression was one of circular thallus form. A filamentous growth habit is produced by division of the peripheral cells. The branching is partially dichotomous, partially sympodial in form (described as pseudo-dichotomous by Fritsch, 1959). As growth continues, calcification takes place in the oldest cells first, forming a thallus of calcified inner portions and growing outer cells (Figure 39).

Results

Growth rate.

The growth of coralline algae in the laboratory was determined by periodically measuring the thallus diameter on the glass sides of two aquaria. The first aquarium contained coralline encrusted bottles which produced the measured thalli. The thalli were tentatively identified as Melobesia membranacea by the numerous lateral cell fusions and lack of megacells. The aquarium was located in a basement laboratory with little light except from overhead fluorescent lighting. Measurements were taken for 26 days, then ceased as growth tended to become polystromatic instead of peripheral. The mean and range of diameter for each of three sides of the aquarium, with a curve to fit all three, is shown in Figure 12.
Figure 9
Photomicrograph of filamentous coralline growth without megacells, taken with polarized light. X 100.

Figure 10
Photomicrograph of disc- or fan-shaped coralline growth with a fringe-like thallus periphery. X 100.

Figure 11
Photomicrograph of fan-shaped coralline growth. X 100.
Growth of coralline algae in the laboratory showing mean and range of diameter for thalli on three sides. (Side A, 11 thalli; side B, 4 thalli; and side C, 5 thalli.) One curve is drawn to fit the three sets of data.
The second aquarium contained *Porolithon gardineri* heads which produced the thalli measured for growth. The growth data were collected for 36 days and are presented in Figure 13. An explanation for the differences in growth of these algae may be given by variations in light intensity on the aquarium sides. The aquarium was placed in such a way that natural light was received from three sides and artificial light from the fourth side and overhead (Figure 14). The location of another aquarium next to one side served as a filter. Foot-candle readings were taken at three intervals during the day and when artificial light was used. The results are given in Table 2.

<table>
<thead>
<tr>
<th>Table 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light intensity (foot-candles) on aquarium sides* used for measuring growth rate of coralline algae (Figure 14).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Side</th>
<th>Daylight 9:30 AM</th>
<th>Daylight 1:30 PM</th>
<th>Daylight 4:30 PM</th>
<th>Artificial light</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>250</td>
<td>100</td>
<td>35</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>300</td>
<td>150</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>175</td>
<td>500</td>
<td>200</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>120</td>
<td>500</td>
<td>150</td>
<td>10</td>
</tr>
</tbody>
</table>

* Average of readings taken over the entire side.

It can be seen in Figures 13 and 14 that growth rate and number of spores settling are inversely related to the light intensity. Because, in nature, increase in light intensity is correlated with increase in ocean temperature, studies were done on the relationship of temperature and light to coralline growth rates. Pieces of
Figure 13

Growth curves of coralline algae grown in different light conditions (see Figure 14 and Table 2) showing mean and range of diameter for four sets of data. (Side 1, 20 thalli; side 2, 21 thalli; side 3, 3 thalli; and side 4, 1 thallus.)
Figure 14

Location of aquarium in laboratory with respect to incident light and distribution of coralline thalli.
DISTRIBUTION OF CORALLINE THALLI
SIDES OF AQUARIUM, EXTERIOR VIEW
plastic were placed in the ocean for 27 days to allow coralline algae to settle. These were brought into the laboratory and placed in aquaria. Growth of the algae was recorded under conditions involving two temperatures of sea water, 18.5°C and 22.5°C, and two light intensities, daylight and artificial light. The results are presented in Figure 15, and show that the sea water temperature has a greater influence on growth than the light intensity. However, it was already demonstrated in Figure 13 that light intensity adversely affects growth. Furthermore, observations on coralline growth occurring on an aquarium in the laboratory showed that most abundant settling took place along an area shaded by a window upright in preference to full window light (not direct sun).

Effects of illumination under natural conditions (i.e., in the field) were investigated in conjunction with substrate color preference. Holders containing glass cover slips with painted backs (clear, red, yellow, green, blue, and white) were attached to a concrete block and placed in the ocean for 14 days to allow settling of algae. The position of the concrete block and holders was noted in reference to compass direction, wave surge, and the path of the sun (Figure 16). The settling of algae on the holders is presented in Figure 17. It was found that the largest thalli occurred with exposure direction of lowest radiation (north, east and west), while the number of thalli appeared to be related to surge produced by the breakers. The substrate color was an indirect test of absorption and reflection of light. With white as a background, less coralline growth and smaller thalli were observed.
Growth curves of coralline algae in two light intensities at two temperatures showing mean and range of thallus diameter. (Direct light at 18.5°C, 13 thalli; indirect light at 18.5°C, 8 thalli, upper curve, and 7 thalli, upper curve, and 10 thalli, lower curve; and indirect light at 22.5°C, 10 thalli for both upper and lower curves.)
Position of concrete block and cover slip holders in reference to compass direction, wave surge and the path of the sun. See Figure 17 for distribution of corallines on the holders.
Figure 17

Distribution and size of coralline algae growing under different conditions of substrate color and exposure direction. (See Figure 16)
Because the effects of light on coralline growth were clearly indicated (Figures 13, 15 and 17), it was decided to investigate cellular changes employing electron microscopy. *Porolithon gardineri* heads and cover slip corallines were brought into laboratory aquaria and kept in continuous dark, continuous light, and normal day conditions for 24 hours. When the cover slips with young coralline algae were fixed with cold aqueous BaMnO₄ (which stains polysaccharides as well as giving good fixation for electron microscopy), the differences in staining were distinctly visible in light micrographs (Figure 18). It can be seen that the normal day corallines were stained deeply by BaMnO₄ in a band approximately two cells wide, three to five cells from the thallus periphery. The 24 hour light corallines stained in the same area but less intensely, while the 24 hour dark corallines stained the lightest but in a wide band of four to five cells. The effects of light on the cell are presented in the section on ultrastructure. When the heads of *P. gardineri* were kept in the dark for 24 hours, a much deeper pink pigmentation was observed than in the normal day or 24 hour light conditions.

Cellular growth rate and pattern were determined by periodical photographic records of coralline thalli growing on glass cover slips. Representative growth rates and patterns from the thalli are shown in Figures 19 to 22.

A comparison of the growth rates from cell counts and the diameter measurements is presented in Table 3. The number of cell rows added per day was converted to diameter:

\[
\text{no. of cell rows/day} \times \frac{\text{average cell length (20µ)}}{2} = \text{diameter increase}
\]
Figure 18

Photomicrographs of cover slip corallines stained with BaMnO$_4$. The effect of continuous light is shown in A, resulting in deep staining at the thallus margin and scattered up to 5 cells deep. In B, normal light conditions produce deep staining in a narrow band 2-3 cells from the margin. Continuous dark (C) results in a less intense and a broader staining region, starting from 2 cells submarginal to as many as 8 cells wide. The regularly spaced dark dots in all of the photomicrographs are the deeply staining cover cells.

A, B, and C: X 200.
Figure 19

Cellular growth pattern of coralline alga traced from photomicrographs taken at 36 hours intervals. The sequence of measurements is: solid line, original thallus, 6 days, 10.5 days and 15 days; dotted line, 2 days, 7.5 days, 12 days and 16.5 days; and dashed line, 4.5 days, 9 days, and 13.5 days.

Thallus l. X 200.
Figure 20 (above) and Figure 21 (below).

Cellular growth patterns of coralline thalli traced from photomicrographs taken at 36 hour intervals. The sequence of measurements is the same as for Figure 19. Figure 20 shows thallus 3 of Figure 22. Both figures X 200.
Growth curves calculated from the increase in number of rows of cells in the coralline thallus. Thalli 1 and 3 are shown in Figures 19 and 20, respectively.
Table 3
Comparative Growth rates of Coralline Algae Determined by Cell Count and Thallus Diameter Measurement

<table>
<thead>
<tr>
<th>Algal Description</th>
<th>Measure of Growth</th>
<th>Substrate</th>
<th>Estimate of Light Intensity</th>
<th>u/Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porolithon gardineri in aquarium</td>
<td>diameter</td>
<td>glass</td>
<td>medium</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>medium</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>bright</td>
<td>30</td>
</tr>
<tr>
<td>Corallines from ocean rows of cells</td>
<td></td>
<td>glass</td>
<td>medium</td>
<td>85</td>
</tr>
<tr>
<td>(not M. membranacea)</td>
<td>cells</td>
<td></td>
<td>medium</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>medium</td>
<td>50</td>
</tr>
<tr>
<td>Melobesia membranacea in aquarium</td>
<td>diameter</td>
<td>glass</td>
<td>dim</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>dim</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>dim</td>
<td>100</td>
</tr>
<tr>
<td>Corallines from ocean on plastic pieces</td>
<td>diameter</td>
<td>plastic</td>
<td>medium</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>medium</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>medium</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>medium</td>
<td>30</td>
</tr>
</tbody>
</table>

In Table 3 it can be seen that *P. gardineri* thalli growing on an aquarium side compare closely to the growth rate of corallines on glass cover slips under comparable light conditions. From this correlation of growth rates, it would appear that gross thallus measurements in millimeters is as valid as actual cell count. The lower growth rate with increased light is consistent with previous experimental data (Figures 13, 15 and 17). *Melobesia membranacea* has the highest growth rate, but whether this is due to genus or to the dim light cannot be ascertained from the experiment. The corallines growing on plastic pieces disclose a growth rate approximately 0.5 the growth rate of corallines on glass cover slips under identical conditions of settling and laboratory maintenance (Table 3). Experimentation with plastic and glass cover slips for coralline settling
indicated that most green and brown algae were epiphytic on corallines growing on glass cover slips. Only a disc-like green alga, probably \textit{Pseudulvella} sp. (\textit{Chaetophoracea}), commonly grew directly on the glass. However, on plastic cover slips there was an abundance of non-coralline growth, especially the grown alga \textit{Sargassum}. The lower growth rate of corallines on plastic seen in Table 3 could be the result of either competition for space on the cover slip (rubbing with the fingers did not remove non-coralline growth as on a glass surface) or to the non-calcareous nature of the substrate.

\textbf{Ultrastructure}

It was pointed out earlier that for clarity in understanding calcification, general coralline ultrastructure would be presented. Electron micrographs of sections from a fragment of heavily calcified \textit{Porolithon gardineri}, sectioned in an undetermined plane, are shown in Figures 23 and 24. The dense calcite can be seen surrounding the cells. Sections from corallines embedded from cover slips and sectioned parallel to the cover slip are shown in Figures 25 to 29. These figures show the effect of light conditions on the cell ultrastructure after being kept in constant dark, constant light, or normal day conditions for three days. The constant dark thallus appears very degenerate and probably had died. The constant light thallus has smaller starch granules and more rounded chromatoplasts, in comparison to the elongate chromatoplasts and larger starch granules of the normal thallus.

Sections of corallines embedded from cover slips and sectioned in a plane perpendicular to the cover slip are shown in Figures 30
Figure 23
Electron micrograph of heavily calcified coralline, sectioned in an undetermined plane. The calcite crystals have fallen out of an extensive matrix, om, leaving open areas. Cover cells, cc, with dense lamellae, chromatoplasts, Cp, and epiphytes, e, along the algal periphery, p, can be seen. X 3,400. Glutaraldehyde-OsO₄-Pb citrate.
Figure 24

Section of heavily calcified coralline alga showing crystals, cr; organic matrix, om; cell wall, cw; chromatoplasic, cp. The white areas scattered in the mineralized region indicate where crystals have fallen out of the section. Gluteraldehyde-OsO₄-Pb citrate. X 14,300.
Figure 25

Low magnification electron micrograph of the coralline periphery, sectioned parallel to the direction of growth and showing no evidence of mineralization. Cover cells, cc, present a zebra-like appearance; nuclei, n; chromatoplasts, cp; cell wall, cw; starch granules, sg; mitochondria, m; central cell vacuoles, cv; and the thallus periphery, p, are noted. BaMnO₄-Pb citrate. X 3,500.
Figure 26

Electron micrograph of a coralline sectioned parallel to the direction of growth in the uncalcified periphery. The alga had been kept in continuous light for 3 days prior to fixation, causing a swelling of the chromatoplasts, cp, and smaller, more numerous starch granules, sg, than occur under normal conditions (Figure 28). Pit connections, pc; lateral cell fusions, lf; nuclei, n; mitochondria, m; central cell vacuoles, cv; cell wall, cw. BaMnO₄-Pb citrate. X 5,700.
Figure 27

Section parallel to direction of growth of the uncalcified periphery of a coralline kept in continuous dark for 3 days. The chromatoplas, cp, degeneration is apparent, as well as other organelles. A pit connection, pc, between a cover cell, cc, and lower cell is present. BaMnO₄-Pb citrate. X 10,000
Figure 28

Section parallel to growth of the uncalcified coralline periphery after normal light conditions. Chromatoplasts, cp; nuclei, n; starch granules, sg; mitochondria, m; central cell vacuoles, cv; cell wall, cw.

BaMnO₄-Pb citrate. X 8,000.
Figure 29

Section parallel to growth of the uncalcified periphery of a normal coralline alga showing cell organelles especially well. Cover cell, cc; nuclei, n; chromatoplasts, cp; mitochondria, m; Golgi body, g; lipid bodies, lb; cell wall, cw; pit connections, pc; starch granules, sg; endoplasmic reticulum, er; thallus periphery, p. Ba$_4$MnO$_4$-Pb citrate. X 11,000.
and 31. These electron micrographs show the relation of the cover cell (zebra-like) to the lower cells especially well. These sections, and the preceding ones of cover slip algae, are taken near the outer edge of the thallus and show no calcification yet. The organelles and structures commonly found in the cells of coralline algae are given on the electron micrographs.
Figure 30

Low magnification electron micrograph of a coralline sectioned perpendicular to the direction of growth. Cover cells, cc, with dense lamellae are situated above the lower cells with chromatoplasts, cp; starch granules, sg; and the very edge of a pit connection, pc. Glutaraldehyde-Pb citrate. 
X 7,000.
Figure 31

Higher magnification of a coralline sectioned perpendicular to the direction of growth. Pit connections, pc, connect a cover cell, cc, to a lower cell and two lower cells together. Nuclei, n; chromatoplasts, cp; mitochondria, m; starch granules, sg; cell wall, cw. The lower edge was attached to the cover slip. Gluteraldehyde-Pb citrate. X 21,500.
CHAPTER III

Crystal Orientation and Description

Introduction

As a preliminary to study of deposition processes in the coralline algae, attention was given to crystal composition and form, using X-ray diffraction and spectrometry techniques. Most mineralized tissues contain depositions of calcium. In the majority of invertebrates and many plants, the calcium occurs in one of the three crystalline polymorphs of calcium carbonate, aragonite, calcite and vaterite (Tunell and Murdoch, 1959; Dana's Manual of Mineralogy, 1966) (Figure 32). The polymorph formed is affected by many factors, including taxon, ions, enzymes, matrix, temperature, and precipitation rates (Kitano and Hood, 1965). Aragonite changes form to calcite when kept in contact with the supernatant (Bachra, Trautz and Simon, 1963) or under pressure (Pankiwskyj, 1968). Vaterite is an unstable crystalline form of the hexagonal-rhombohedral division and rarely occurs naturally (Wilbur and Watabe, 1963). Calcite is the most commonly occurring polymorph of calcium carbonate, deposited; for example, by bacteria (Wainwright, 1970), foraminifera (Be and Ericson, 1963), sponges (Travis, 1970), crustaceans (Travis, 1970), some molluscs (Wilbur and Watabe, 1963), echinoderms (Hyman, 1955; Boolootian, 1963) and in the avian egg shell (Schmidt, 1966; Schraer and Schraer, 1970). Coral and some molluscs deposit aragonite. Mixtures of the calcium carbonate polymorphs are rare in plants, but in the mussel (and many Pelecypods), aragonite and calcite are deposited in adjacent layers, the nacreous and prismatic regions,
Figure 32

General structure of two crystalline polymorphs of calcium carbonate (after Tunell and Murdoch, 1959).

A. Aragonite - general orthorombic crystal form of the rhombid-dipyramidal class

\[
\begin{align*}
\frac{2}{m} & \frac{2}{m} & \frac{2}{m} \\
\end{align*}
\]

B. Calcite - general hexagonal-rhombohedral form of the hexagonal-sclenohedral class

\[
\begin{align*}
\frac{3}{m} & \frac{2}{m} & 1 \\
\end{align*}
\]
respectively (Travis, 1970). More than one crystal form also may occur under specific incubation conditions (Wilbur and Watabe, 1963) and in regenerating shells of both aragonitic and calcitic molluscs (Wilbur and Watabe, 1963) and an aragonitic gastropod (Muzii and Skinner, 1966).

Vertebrates commonly deposit calcium phosphate as highly crystalline hydroxyapatite in tooth enamel (Johnson, Armstrong, and Singer, 1966) or as a nonapatitic, amorphous calcium phosphate precursor to hydroxyapatite in bone (Budy, 1966). Calcium oxalate crystals are found intracellularly in many plants, including water hyacinth, Ginkgo (Arnott and Pautard, 1970) and Geraniaceae (Kinzel, 1963).

From their investigations, Baas-Becking and Galiher (1931) concluded that calcite was the only mineral deposited by the coralline algae, and that the c-axis of the crystal was perpendicular to the longitudinal axis of the fibres. These data have been confirmed by subsequent investigations, with the additional finding that magnesium carbonate (as magnesian calcite) is commonly present in amounts of 12-14 mol-% and ranging up to 30 mol-% (Chave, 1957; Noberly, 1968). The coralline Goniolithon sp. has a high-magnesian calcite skeleton consisting of a physical mixture of two crystalline phases, the dominant phase being magnesian calcite and perhaps a lesser phase of magnesium hydroxide (brucite) Schmalz, 1965; Weber and Kaufman, 1965). There also occurs in the Corallinaceae a non-calcifying alga, Schmitziella endophloea, which is an endophyte of Cladophora (Suneson, 1944).

Not all mineralizing algae deposit the calcite form of calcium carbonate, however. The red alga Liagora (Nemalionales) deposits
aragonite (Lewin, 1962), as do the brown alga Padina (Lewin, 1962) and all the green algae (Chave and Wheeler, 1965). The golden-brown (Chrysophyceae) alga *Coccolithus huxleyi* (also known as the protozoan *Coccolithophorida*) normally forms calcite and traces of aragonite and calcium phosphate. But a nitrogen-deficient medium induces vaterite formation along with calcite and aragonite, with temperature affecting the relative crystal amounts (Wilbur and Watabe, 1963).

Because of the variety of skeletal minerals found in organisms, it was deemed necessary to verify the composition of the coralline (cell wall) as calcium carbonate by X-ray spectrometry and determine the crystal polymorph by X-ray diffraction. After this was done, polarized light microscopy was employed to demonstrate calcified areas, identified by the high birefringence of calcite (Hartshorne and Stuart, 1960). Calcification in the green alga *Halimeda* was described by Wilbur, Colinvaux, and Watabe (1969) "Successively older segments, up to a certain stage of maturity, show increasing degrees of calcification." This age-calcification correlation agrees with the generally accepted description of a calcified inner and viable, non-calcified outer thallus in the coralline algae. However, the actual progression of calcite deposits within a coralline was never ascertained, only the final situation noted. Because of this lack of information, samples were studied from various locations on a *Porolithon gardineri* branch for arrangement of calcified cells.

Earlier studies of crystal orientation in coralline algae emphasized the crystal direction in relation to fibre and whole thallus structure, with no investigations performed on a cellular
level. In the present studies, orientation of crystals was determined by electron diffraction and sectioning of calcified corallines for electron microscopy. Thus, orientation and density of crystals could provide information for further studies on time and location of crystal precipitation. Less dense crystals could be assumed to be more recently formed than thick aggregates, and orientation patterns could be regarded as implicating involvement of an organic matrix.

Investigations on a cellular level, especially replications for electron microscopy, have produced a great deal of information on crystal orientation in calcifying organisms. More electron microscopy has been done on the molluscan shell than any other mineralizing system. In the mussel *Mytilus* (Pelecypoda), calcite is deposited in prisms arranged in ordered, close-packed sheets (Travis, 1970). Subjacent to the prismatic layer is the nacreous layer, the pearly inner shell, with tabular aragonite crystals in horizontal lamellae (Watabe, 1965). The gastropod nacre is formed of aragonite stacks, increasing the number of crystals that can develop at one time (Wise, 1970). Aragonite spherulites were initially deposited on a nail-polish membrane which induced shell repair in an aragonite gastropod, with the normal lamellar shell structure restored later (Mizii and Skinner, 1966). In brachiopods, calcitostracum, a shell layer of lamellar structure similar to pelecypod nacre, is formed from calcite (Towe and Harper, 1966). The avian egg shell reveals the same sequence of layers as found in the pelecypods, though oriented radially instead of tangentially (Heyn, 1963). Small crystallites form at the organic matrix and grow into oriented lamellar crystals of the nacreous layer.
Of unicellular organisms, the foraminifera have been widely used to study crystal development and orientation. Small, thorny crystals, called "punctae" by Be and Ericson (1963), were the earliest indication of calcite development in foraminifera. These punctae were found on top of the original bilamellar test, which consists of an outer layer of calcite rhombohedrons and an under layer of randomly oriented, earlier calcite crystals (Lynts and Pfister, 1967), and buried in large crystals in the calcite crust (Be and Ericson, 1963). Polarized light pointed out the preferred calcareous orientation of crystals in the granular wall species of foraminifera and the random crystal array in species with porcelainous walls (Towe and Cifelli, 1967). Other electron microscopy studies on mineralization in unicellular organisms have shown membrane-bound mineral deposits within cells in the ciliate *Prorodon morgani* (Andre and Faure-Fremiet, 1967) and in diatoms (Reimann, 1964). The coccolith is also formed within a membrane-bound area, and electron diffraction of a coccolith of *Coccolithus huxleyi* revealed a single calcite crystal, with the c-axis parallel to the direction of elongation (Watabe, 1967).

Crystallographic studies on orientation and morphology of echinoderm calcite revealed that nearly all the plates and spines are single crystals with different orientation in different species (Nissen, 1969). However, Towe (1967) believes that the plate exterior is a "polycrystalline aggregate with preferred orientation" and suggests an initial oriented, polycrystalline growth followed by recrystallization and fusion into one single crystal. Scanning electron microscopy showed rounded crystal surfaces, "mammillate structure", and conchoidal fractures of the plates, unusual for calcite which
normally has sharp cleavage faces (Nissen, 1969).

Turkey leg tendon first deposits mineral with the c-axis parallel to the collagen fibril axis (Nylen, Scott and Mosley, 1960). These first crystals appeared to be platelets and filling in occurred by the deposition of needles. In bone, the early stages showed no preferred orientation, but compact bone had the apatite c-axis parallel to the collagen fibre axis (Eanes and Posner, 1970). Octacalcium phosphate, calcium-deficient apatite, is converted to hydroxyapatite as aging (hydrolysis) occurs, resulting in different bone mineral at different stages of development (Budy, 1966).

In plants, mineral may be found as intracellular depositions in vacuoles and on, or within, cell walls. Crystalloplastids are a special feature of some crystal cells in which calcium oxalate is found (Arnott and Pautard, 1970). These plastids contain a dense zone with small, ordered membranes and a zone of thylakoid-like membranes and apparently arise from proplastids in *Yucca*. In *Zygocactus*, chloroplasts are modified into crystalloplastids and contain irregular zones with dense, crystalline inclusions. Aragonite crystals are first formed at the filament wall in *Halimeda*, with the intertriccular spaces eventually filling with crystals of random orientation (Wilbur, Colinvaux and Watabe, 1969).

In extensive electron microscopy done on calcified coralline fragments and cover slip thalli, crystal orientation was readily apparent, and metal shadowing of the sections elucidated crystal shape. Three different orientation patterns and two crystal forms were shown in thin sections. Intensive calcification parallel to the direction of growth, small crystals oriented perpendicular to the
plasmalemma and coalescing into larger crystals further from the cell, and fibrous, random intracellular crystallization could all be seen in one micrograph (Figure 33).

**Results**

**Crystal composition**

To analyze crystal composition, X-ray fluorescent spectrometry of pulverized material from *Porolithon gardineri*, encrusting coralline algae, and *Halimeda discoidea*. Powders of 100 to 200 mesh were obtained by grinding the dried algae in a mortar and pestle, then sieving the powders and placing the fine material on slides for analyzing. In addition, thalli of the brown alga *Padina japonica* were dried directly onto the glass slide. *Halimeda* and *Padina* were used as controls because they are known to contain calcium carbonate in the form of aragonite (Lewin, 1962), allowing less substitution for calcium in the crystal lattice (Chave, 1957). Samples were scanned with a tungsten tube and an ADP (ammonium diphosphate) crystal. In order to use a longer wave length to detect magnesium, the ADP crystal was substituted for the lithium fluoride crystal normally used. However, the upper limit of ADP (10.0 Å) may be too close to detect magnesium (Kα, 9.9 Å and Kβ, 9.5 Å), accounting for the lack of magnesium recorded. Spectrographic records of the four algal specimens are shown in Figure 34. The calcium peaks are indicated on the recordings. These correspond to the following K X-ray lines of calcium:
Electron micrograph of heavily calcified coralline alga sectioned perpendicular to the direction of growth showing the three crystal sites - intercellular, 1, cell edge, 2, and intracellular, 3, - and two crystal types - tabular, T, and fibrous, F. Glutaraldehyde-Pb citrate-BaMnO₄. X 18,200.
Figure 34

X-ray spectrometer recordings of four algal powders showing the $K\alpha$ and $K\beta$ X-ray lines for calcium.
Figure 34

X-ray spectrometer recordings of four algal powders showing the Kα and Kβ X-ray lines for calcium.
Crystal structure

To verify that the crystal form of calcium carbonate in the coralline algae is calcite, some diffraction by X-rays was conducted. The same four algal samples and preparation were used as for X-ray spectrometry. For comparison of diffraction patterns with the preparations, known calcite and aragonite crystals were obtained from the Hawaii Institute of Geophysics and served as diffraction standards. The diffraction records of the four calcareous algae are shown in Figure 35. The peaks for identification of crystal structure are indicated. For purposes of comparison, the three most intense X-ray diffraction peaks of calcite and aragonite are:

<table>
<thead>
<tr>
<th>Crystal</th>
<th>d-value (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>calcite</td>
<td>3.04 2.29 2.10</td>
</tr>
<tr>
<td></td>
<td>(30.2) (40.3) (44.0)</td>
</tr>
<tr>
<td>aragonite</td>
<td>3.40 3.27 1.98</td>
</tr>
<tr>
<td></td>
<td>(26.4) (27.5) (45.9)</td>
</tr>
</tbody>
</table>

* The value for degrees 2θ for Cu K radiation is given below in parentheses.

It is easily seen from the recordings that calcite is the crystal form of calcium carbonate found in the corallines studied, while aragonite occurs in Padina and Halimeda.
Figure 35

X-ray diffraction patterns of four algal powders. The major peaks are labelled 'aragonite' and 'calcite'.
The three identifying peaks of calcite and aragonite are labelled by the crystal name, while lesser peaks are labelled by initial from data obtained from Wilbur and Watabe (1963). The tall peaks at 32°, 46° and 57°, 2θ for Halimeda and other on algae is probably due to NaCl in the algal powder.

Dr. Hans-Ude Nissen of Eidgenossische Technische Hochschule, Zurich, very kindly recorded the same series of four algae, Porolithon gardineri, encrusting coralline, Halimeda discoidea and Padina japonica, with an X-ray diffraction powder camera. The diffraction results are presented in Figure 36. It can be seen that these are the same as Figure 35, except for one important difference. Dr. Nissen's results show both calcite and aragonite occurring in the encrusting coralline. A mixture of these two crystal forms had never been found in red algae before, though not uncommon in other organisms, e.g., mussel (Travis, 1970). It was not possible to reproduce the results obtained by Dr. Nissen. This important difference raised many questions, but several possibilities may be considered as explanations for the inconsistency. 1) The aragonite may have been from a fraction of coral substrate that was chipped off with the coralline. 2) The aragonite changed into calcite by the time the diffraction was repeated. 3) A piece of molluscan shell may have been included. 4) Only a very small part of the encrusting coralline contained aragonite (new growth on the surface) and that part was removed in the first diffraction. The question was not pursued further in the present study, and the results of the recording in Figure 35 will be considered in later discussion.
Figure 36

X-ray powder diffraction patterns of four algal powders. The three major diffraction lines for aragonite, A, and calcite, C, are indicated.

(Courtesy of Dr. Hans-Ude Nissen.)
Diffraction of algal powders was also done with an Hitachi HU-11A electron microscope. Samples of the same powders used for X-ray diffraction were glued to copper grids and diffracted with electrons. While no patterns of molecular structure were obtained, powder rings, two showing orientation of the crystals, were recorded. Representative diffraction patterns (two showing crystal orientation) are shown in Figures 37 and 38.

**Progressive calcite deposition.**

Geological thin sections were cut from a branch of *Porolithon gardineri* to survey the amount and location of calcite deposition within cells. Cross sections and longitudinal sections were taken from the distal tip to proximal base of one 6mm branch of *P. gardineri*. The thin sections were examined in the microscope with or without polarizing lenses. Photomicrographs of representative cross sections under polarizing lenses are shown in Figure 39. The longitudinal sections were similar, but several microscope fields were necessary to visualize the pattern. It can be seen in these micrographs that the dark (non-calcified) cells progressively increase in number toward the distal portion of the branch, and that completely calcified cells appear in the center of the branch first. Areas of intense birefringence were seen in sections approximately half-way between the distal and proximal portions of the branch. These areas occurred at locations bridging complete calcification and semi-calcified regions. The areas are difficult to perceive without scanning the section in the microscope, but an idea of their location and structure can be seen in Figure 40.
Figure 37

Electron diffraction patterns of a calcite standard (A), an encrusting coralline (B), and the carbon-coated collodion film used to coat electron microscope grids (C).
Electron diffraction patterns of encrusting corallines, showing preferred orientation of the calcite crystals by the symmetrical spots in A and the bright areas, symmetrically arranged, on the diffraction rings in B.
Figure 39

Photomicrographs of *Porolithon gardineri* branch sections taken with polarized light, showing more complete calcification (birefrigence) in the more mature sites. Geological thin sections were prepared from the areas indicated on the diagrammed branch.

All photomicrographs X 125.
Figure 40

Photomicrograph taken with polarized light showing intensely birefrigent areas scattered in *Porolithon gardineri* branch. X 200.
**Electron microscopy**

The heavily calcified sections of a coralline alga show the crystal orientation in two directions (Figure 41). Crystals are oriented perpendicularly to the cell, at a point well within the cell wall. Other crystals are orientated parallel to the direction of growth and extend to the distal margin, sometimes encircling a cover cell (Figure 42 and 43). Crystal location along the cell wall and in reference to a cover cell is shown in Figure 44. In this figure, crystals can be seen to be along an electron dense layer of the cell wall, oriented at right angles to the wall. It can also be seen that crystals have formed beneath the dense layer in some regions. In Figure 45, three stages of calcite deposition are presented. It has been found that at the cover cell, near the algal periphery, this formation is lacking (Figure 41 and 46). It can be seen in Figure 46 that the crystals are parallel or randomly oriented as they encircle the cell.

In some sections with the oriented crystals (Figure 47), fibrous intracell elements are seen. The corallines, embedded from coverslips, have epiphytic growth, and from the lack of cellular organization, it appears that the alga is dead. The fibrous crystals extend inward from the cell wall and occur at intracellular sites. Figure 48 shows the crystal orientation within the fibres, occurring as electron dense areas between fibrous edges extending into the cells.

Incompletely calcified sections of coralline fragments show separation of the oriented crystals into two distinct areas (Figures 49 and 50). One location is at the inner cell wall, and the other location is interfilamentous, if the coralline is visualized as being
Figure 41
Section of heavily calcified coralline alga showing the orientation of crystals. Small perpendicular crystals are located at the lower cell edge (open arrow). An extensive organic matrix can be seen surrounding the crystals. Crystals are oriented parallel to the cover cell and to the direction of growth (solid arrow). Cover cells, cc; chromatoplasts, cp; lipid bodies, lb. Glutaraldehyde-OsO_4-Pb citrate. X 20,800.
Figure 42

Low magnification electron micrograph of a heavily calcified coralline showing the orientation of crystals and the extensive organic matrix left where crystals have fallen out of the section. The area within the square is shown at higher magnification in Figure 43. Thallus periphery, p; organic matrix, om; crystals, cr; chromatoplasts, cp; pit connections, pc; starch granules, sg. Glutaraldehyde-OsO₄-Pb citrate-BaMnO₄. X 6,000.
Figure 43

Enlargement of area within the square in Figure 42 showing crystal orientation and the organic matrix structure. Thallus periphery, p; organic matrix, om; cell wall, cw. Glutaraldehyde-OsO₄-Pb citrate-BaMnO₄. X 11,600.
Figure 44

Section of a calcified coralline perpendicular to the direction of growth showing crystal orientation along the cell wall and in reference to a cover cell. Some crystals can be seen to have formed within the cell wall (arrow). Thallus periphery, p; cover cell, cc; cell wall, cw; chromatoplast, cp. Glutaraldehyde-OsO₄-Pb citrate. X 15,000.
Three progressive stages of calcite deposition in coralline algae. A shows the small crystals next to the cell and larger ones intercellularly. In B, the crystals next to the cell have grown larger, and there is denser calcification. Large crystals have formed next to the cell and intercellularly in C, with little uncalcified area left next to the cell wall. Gluteraldehyde-OsO₄-Pb citrate (and BaMnO₄ in C). All electron micrographs at X 13,000.
Figure 46

Section through a cover cell at the coralline periphery showing crystal orientation and location. The crystals are parallel to or randomly oriented around the cover cell. Thallus periphery, p; cover cell, cc; organic matrix, om. Glutaraldehyde-OsO₄-Pb citrate-BaMnO₄.

X 10,400.
Section of a degenerate coralline perpendicular to the direction of growth showing the fibrous intracellular crystals, F, and tabular intercellular crystals, T. Gluteraldehyde-Pb citrate-BaMnO₄. X 9,000.
Figure 48

Section of a coralline perpendicular to growth direction showing fibrous areas. Fibrous elements extend into the cell (F), and electron dense areas (arrow) occur within the thick fibrous intercellular regions (DF). Glutaraldehyde-Pb citrate-BaMnO₄. X 23,700.
Figure 49

Section of a coralline fragment showing crystals in two distinct areas—the inner cell wall and intercellularly. (Stained for acid phosphatase.) The area within the square is shown at higher magnification in Figure 50. Chromatoplasts, cp; cell wall, cw; pit connections, pc. Glutaraldehyde-Pb(OH)$_2$. X 6,700.
Figure 50

Higher magnification of square area in Figure 49, showing perpendicular orientation of crystals at the cell edge and parallel orientation of the intercellular crystals. (Acid phosphatase stained.) Chromatoplast, cp; cell wall, cw. Gluteraldehyde-Pb(OH)$_2$. X 21,200.
composed of cells in closely apposed filaments extending peripherally. The tabular crystal shape is demonstrated in the interfilamentous calcification, some crystals oriented on their sides and some flat (Figure 51). The crystals are approximately 300-600\(\AA\) wide, with varying length, before coalescing. "Sheaths" are seen in some areas, appearing to be intracellular and measuring approximately 300\(\AA\) wide (Figure 52). In another micrograph from the same section (Figures 53 and 54), there appears to be an area with fibrous crystals.

The first indication of mineral precipitation was found immediately exterior to the plasmalemma (Figures 55 and 56). Periodicity of 150\(\AA\) can be seen within the deeply staining interior cell wall. This first nucleation occurred approximately 50 cells (0.5 mm) from the coralline thallus periphery. The crystallization shortly appeared to look like stacks of pennies. These stacks occur at approximately 150\(\AA\) intervals and are shown as they appear laterally to the cell (Figures 55 and 57) and on top of a cell (Figures 55 and 58).

The periphery of a coralline alga is presented in Figure 59. The absence of any calcium carbonate crystals is apparent. Cell wall changes are commented upon in the discussion of the organic matrix.

Crystal form was explored by shadow casting grids holding sections previously observed in the electron microscope. Pieces of coralline algae were scraped off a plastic holder which had been left in the ocean for 20 days. The crystal shape is shown to be rounded (Figure 60), without the sharp cleavage faces expected from the unshadowed section (Figure 61). Crystal orientation is enhanced,
Section of a coralline alga with the tabular crystal shape illustrated. Crystals can be seen flat, sideways and sections across the length (especially apparent at top right). Acid phosphatase stained.
Gluteraldehyde-Pb(OH)$_2$. X 22,700.
Figure 52

Section of a coralline fragment showing crystal "sheaths" of approximately 300Å width (at arrow). (Lower magnification of this figure and Figure 54 is given in Figure 53.) (Acid phosphatase stained.) Gluteraldehyde-Pb(OH)_2. X 21,500.
Figure 53

Low magnification electron micrograph of a coralline alga showing the areas of "sheath" location (A) and probable fibrous crystals (B). (Acid phosphatase stained.) Gluteraldehyde-Pb(OH)₂. X 7,300.
Section of coralline fragment showing probable fibrous crystals.

F. Lower magnification of this area is given in Figure 53.

(Acid phosphatase stained.) Chromatoplasts, cp.

Gluteraldehyde-Pb(OH)$_2$. X 21,100.
Figure 55

Low magnification electron micrograph of area of first mineral precipitation in the coralline alga. Figure 56 location is indicated by the solid arrow. The approximate locations of Figures 57 and 58 are marked by "top" and "lateral", but the sections for those figures were taken further into the area of calcification. Cover cell, cc; chromatoplasts, cp; starch granules, sg. BaMnO₄, Pb citrate. X 24,800.
Figure 56
Electron micrograph showing the first indication of mineral precipitation in the coralline cell wall (arrow).
(Sectioned perpendicular to direction of growth.)
Periodicity of 150Å is visible within the cell wall.
Chromatoplast, cp; cover cell, cc.
BaMnO₄-Pb citrate. X 91,900.
Figure 57

Section of a coralline perpendicular to the growth direction showing mineral "stacks" at the lateral edge of a cell (arrow). Starch granules, sg. EeMnO₄-Pb citrate. X 70,000.
Figure 58

Section of a coralline perpendicular to growth direction showing mineral "stacks" on the top of a cover cell (arrow). Mitochondria, m; chromatoplasts, cp. BaMnO$_4$-Fe citrate. X 105,000.
Figure 59

Uncalcified coralline thallus periphery, sectioned parallel to the growth direction. Thallus periphery, p; cover cell, cc; chromatoplasts, cp; mitochondria, m; cell wall, cw; nuclei, n; lipid bodies, lb; starch granules, sg; pit connections, pc. BaMnO$_4$-Pb citrate. X 9,800.
Figure 60

Shadowed section of coralline showing the rounded crystal edges. The section before shadowing is presented in Figure 61. Chromatoplasts, cp; pit connections, pc; cell wall, cw. (Arrow indicates shadow angle.) Gluteraldehyde-OsO₄-Pb citrate-Pt/Pd shadowed. X 24,500.
Figure 61
Coralline section before shadowing, with the square enclosing the area of Figure 60. Cell wall, cw; lipid bodies, lb; chromatoplasts, cp; lipid, l; starch granules, sg; pit connections, pc. Gluteraldehyde-OsO₄-Pb citrate. X 10,000.
especially the parallel crystal orientation outside the cover cells (Figure 62). In Figure 63, crystals are seen oriented perpendicularly to the cell, some coalesced into larger units. The crystal pattern between a cover cell and an adjacent cell, including some organic connections between crystals, is presented in Figure 64. The overlapping of inner crystals over outer crystals is well demonstrated in Figure 65.

Discussion

In this study and in previous investigations (i.e. Baas-Becking and Galiher, 1931) calcium carbonate in the form of calcite has been shown to be the only crystal deposited by the coralline algae. Using the X-ray fluorescent spectrometer, calcium was recorded in quantity but not magnesium due to the crystal used for analysis. The lack of magnesium would be most unusual in algal calcite, according to the geoscientists (Chave, 1957; Moerby, 1968). However, Baas-Becking and Galiher (1931) described magnesium deposition in corallines as of secondary importance and sometimes lacking completely, and Fritsch (1959) states that magnesium is only precipitated in older thalli and in dead parts. Because magnesium substitutes for calcium in calcite, it is not of basic importance to this study on crystal deposition whether magnesium is present or not. Therefore, no further effort has been made to ascertain magnesium content in the specimens used in this investigation.

The electron diffraction patterns give evidence of crystal orientation (Figure 38), and, after observing electron micrographs of thin sections, crystal orientation would be expected. The
Figure 62

Shadowed electron microscope section, showing parallel orientation outside the cover cells. Cover cell, cc.
(Arrow indicates shadow angle.) Gluteraldehyde- 
OsO$_4$-Pb citrate-Pt/Pd shadowed. X 45,000.
Figure 63

Crystal pattern in a shadowed coralline section, showing the perpendicular orientation to the cell, large crystal units, and organic matrix. Cell walls, cw. (Arrow indicates shadow angle.) Gluteraldehyde-OsO₄-Pb citrate-Pt/Pd shadowed. × 22,500.
Figure 64

Shadowed coralline section showing crystal pattern and organic connections between crystals (arrows). Cover cells, cc. (Heavy arrow indicates shadow angle.) Glutaraldehyde-OsO₄-Pb citrate-Pt/Pd shadowed.

X 14,000
Figure 65.
Shadowed coralline section showing overlapping of outer crystals by the inner ones in direction of open arrow. (Solid arrow indicates shadow angle.) Cover cell, cc.
Glutaraldehyde-OsO₄-Pb citrate-Pt/Pd shadowed.
X 14,000.
unique, or c-axis, of an hexagonal crystal is generally the long axis. The major portion of the calcified coralline thalli examined had calcite crystals elongated at right angles to the direction of growth. Thus, the finding of Baas-Becking and Galilher (1931) that the c-axis of calcite was perpendicular to the longitudinal axis of the fibre is correlated with crystal deposition at the cell wall. Orientation of the individual crystals in the coralline thallus was impossible to attain on the electron microscopes available for use, because the specimen stage did not rotate to allow identification of the crystal axis diffracted. Diffraction of the discrete crystals for identification and orientation remains to be done.

Crystal growth in the coralline algae had not been investigated prior to this study. Electron microscopy suggests that the initial crystal nucleation takes place within an electron dense cell wall layer which borders the plasmalemma of the cell. This mineralization probably increases in size initially by deposition of calcium carbonate on the crystal nucleus, or seed, creating stacks of crystals. Periodicity of the initial depositions and of the later stacks occurred at intervals of 150Å. The individual calcite crystals ranged from 300-600Å in width, though the discrepancy in measurements may be the result of inaccuracy of calculations from two different electron microscopes. No calibrations were made for the electron microscopes, and magnification was calculated from the intermediate lens voltage. After nucleation, calcification can be postulated to progress in the following way. The crystals appear to be moved away from the cell, coalescing with other crystals until larger units are present in the region between the cells. As crystal growth continues at the cell
edge, the intercellular area is filled with crystals, probably forcing the inner calcite along the direction of algal growth. Larger crystals may be formed at the cell edge when deposition continues faster than the crystals are pushed away or than space is available for crystals to move into. This suggested sequence of crystallization would also account for the transitory occurrence of crystals parallel to the direction of growth. If crystals were first formed parallel to the fibres and to the direction of growth, it would be expected that this crystal orientation would predominate as the calcification spread to the thallus periphery. Instead, it seems that algal growth and the organic matrix may pull the crystals along in the orientation offering the least resistance. Previously mentioned was the possibility of two sites of deposition, at the cell edge and intercellularly. Thus far, no evidence for the intercellular depositions has been found. And the "filament periphery" location of precipitation is more difficult to present than the explanation that growth is responsible for carrying the crystals to the intercellular region. Theoretically, the cells may occur in filaments, but there is no contact with the outside environment at the lateral sides of the cells. The distinct separation of crystal zones in Figures 48 and 54 may be explained as having been sectioned across growth lines which have carried crystals from above or below in the intercellular area. Other investigators, studying the formation of the nacreous layer in molluscs, have proposed similar deposition patterns (Watabe and Wilbur, 1961; Towe and Cifelli, 1967). They propose that seed crystals grow on an organic matrix secreted onto the growth surface. The crystals grow and coalesce to form a layer with growth patterns that may cause
crystals to overlap each other.

The fibrous crystals, or spicules, have been found only in areas of obvious degeneration in the corallines. There is random orientation of the spicules, but oriented electron dense areas are seen associated with the fibres. Whether the fibrous crystals are the result of a purely physical deposition of mineral or an organically influenced precipitation is not known at this time. In corals, the living coenosarc (the hollow internal structure of the polyp) is fairly calcium-proof, preventing equilibrium exchange with the skeleton deposit (Goreau and Goreau, 1960). The non-living coenosarc is not an effective barrier and allows rapid calcium exchange. A similar physiological response may be occurring in the coralline plasmalemma, a living membrane necessary to prevent an equilibrium exchange within the cell. Calcite is known to precipitate out of solution in the form of fibrous crystals (Chave, 1970). The most extensive fibrous elements are found within the cell (Figure 47), with the extremely dense areas occurring intercellularly, where they may be influenced by the organic fibres (Figure 48). Thus, a physical precipitation seems the best explanation for this crystal form. Diffraction of the fibrous area should be done to positively identify the crystal (or fibre).

In investigations with vertebrates where a fibrous crystal form was present, it was identified as hydroxyapatite by diffraction (Hayden, 1970; Schiffman, Martin and Miller, 1970). Schiffman, Martin and Miller found that calcium precipitation occurred in degenerating elastin fibres and that deposition was independent of metabolic factors, suggesting a nucleation phenomenon involving functional groups.
There is a striking resemblance between the spicules occurring in the coralline alga (Figure 47) and those occurring in vertebrates (Figure 66). In tubules from the kidneys of rats treated with Dif-Thorotrast to induce calcification and sacrificed after 120 hours, Hayden (1970) found calcific lesions with associated spicules, which he feels are hydroxyapatite. In the mineralization of rat aorta incubated in serum \textit{in vitro}, calcium and phosphate were found in predominantly hydroxyapatite in the elastin fibres (Schiffman, Martin and Miller, 1970). The minerals were not oriented and had the same appearance as the numerous fibrous crystals in Figure 47. The fibrillar crystals are difficult to measure accurately, but the dimensions can be seen to be comparable in the electron micrographs (Figures 47 and 66).

Physical precipitation would also explain the pattern of cells filled in with calcite found in \textit{Porolithon gardineri} (Figure 39). If it were a physiological occurrence, one would expect a sequential deposition of calcite within cells instead of the rather random mineralization. As calcium carbonate encases a cell, the cell will be isolated from the outside environment and degenerate. With degeneration, conditions are changed to favor physical calcite deposition within an environment saturated with calcium carbonate. Thus, a crystal nucleation of physiological origin grows and orientates within organic influences, with physical precipitation the final step.

The rounded edge of the calcite crystal was apparent in the shadow cast sections. This unusual calcite form appears to be
Figure 66

Section of calcific lesion in the tubule of the kidney of an experimentally treated rat, showing the associated spicules (arrow). Electron micrograph through the courtesy of Dr. Glen Hayden, Stanford Medical Center. Gluteraldehyde-cacodylate, OsO₄, uranyl acetate, Pb citrate. X 42,500.
limited to organic crystals. Nissen (1969) has described the "mammillate structure" of calcite in echinoderms. But the echinoderm structures appeared to be formed by trabeculae (sheets of mesoderm) in fret-like sheets, not individual crystals. In the coralline algae examined in this study, even an isolated crystal on the cell wall exhibited rounded edges. The shadowed sections revealed the inner crystals overlapped the outer ones, providing further evidence for initial formation of crystals at the cell edge and moving out.

In some of the shadowed sections, connections were seen between crystals suggesting an organic component, and in heavily calcified specimens, a fret-work was left when crystals fell out of the section. Organic membranes were seen horizontally connecting crystal stacks in molluscan nacre with scanning electron microscope (Wise and Hay, 1968; Wise, 1970). "Sheaths" were found intracellularly, forming a transition from calcified to non-calcified areas, only in tissue stained for acid phosphatase. The sheaths did not appear to be outlined by a precipitate as would be expected if the acid phosphatase was responsible for their elucidation, so their visibility is probably due to the location and angle of the section. The sheaths were approximately 400Å wide, as were the calcite crystals in the electron micrograph. (The 300Å measurement mentioned above is considered more accurate for the crystal size, having been determined at a higher magnification.)

From the implications of organic involvement obtained from the electron micrographs - an electron dense layer involved in crystal precipitation, "sheaths", fret-work and rounded crystals - it was
decided to undertake further investigations emphasizing the organic matrix.
CHAPTER IV

Histochemistry and Molecular Structure

Introduction

A variety of histochemical investigations were performed to determine constituents of the coralline algae and enzymes that might be important in calcification processes. Comparable tests on calcified and decalcified thalli were used in order to ascertain differences due to crystals covering reactive areas or binding to nucleation sites. Earlier work indicated calcification penetrated the inner cell wall layers after initial deposition in the outer layers (Fritsch, 1959). Using $^{12}\text{C}$ and $^{13}\text{C}$, it was found in some green algae (Halimeda, for one) that the organic matter was enriched in $^{12}\text{C}$ and the aragonite skeletons in $^{13}\text{C}$, indicating a partition into heavy and light components during photosynthesis (Lewin, 1962). Yet in a species of Coralline, both the organic matter and calcite were enriched in $^{12}\text{C}$ compared to surrounding medium, suggesting a process other than photosynthesis involved (Lewin, 1962). Because there appeared to be an active incorporation of calcium, the nature and production of the cell wall provided an initial point for investigation of the processes underlying mineral deposition.

By examining the cell wall (organic matrix) for the presence of amorphous or other structural units, physical and/or chemical binding processes to a structural component could be studied in vitro and in vivo. If the nature of the functional units in the wall were known, the physical processes and specific chemical pathways for making the matrix could be investigated. These could
be through enzyme studies or model systems for mineral precipitation. Finally, the orientation and properties of specific materials and their effects upon optical systems could provide further clues as to the mechanisms most relevant to the organization present.

Previous investigators conflict in their cell wall analyses. Blinks (1951) states that the corallines have cellulose walls, while Fritsch (1959) and Dawes, Scott and Bowler (1961) describe the cell wall as composed of a pectic outer layer and cellulose inner layer. However, Cronshaw, Meyers and Preston (1958) found the cell wall of three red algae (Ptilota, Griffithsia, and Rhodymenia) to consist mainly of glucose units and usually xylose and galactose as well. They found the red alga Porphyra to have only mannose in its cell walls. Polysaccharides composed of glucose, galactose, mannose, arabinose and xylose were found in Porolithon gardineri cell walls by Putman (1965). The conflicting results may be ascribed, in part, to differences in methods. Sugar components of walls were obtained through chemical analyses (Cronshaw, Meyers and Preston, 1958; Putman, 1965); and pectin and cellulose were demonstrated using histochemical technique (Dawes, Scott and Bowler, 1961).

A common structural pattern in the sulfated polysaccharides present in cell walls of the red algae was found by Anderson, Dolan and Rees (1965). The same basic polysaccharide structure was universally present in the red algae studied (not including the corallines): a linear chain of galactose units linked alternately $\alpha$-1,3 and $\beta$-1,4. This basic structure is modified by different algae to produce different polysaccharides (for example, the D-sugar instead of the L-sugar, methylation at different places, and the
anhydride sugar), with sulphation usually found at the 4 or 6 carbon position. Sulphated mucopolysaccharides bound to protein are present in cartilage (Fhavanandan and Meyer, 1966). It was also found associated with sites of calcification in a coccolithophorid (Douglas, Isenberg, Lavine and Spicer, 1967). In coccolithophorids, a role of mineral capture is suggested for the sulphated polysaccharides, because inhibitors of mineralization directly affect the amount of sulphated materials present.

Protein, as collagen, is common in the structural elements of most vertebrate calcification. Wilbur and Watabe (1963) found the amino acid composition of the molluscan matrix influenced the calcium carbonate polymorph laid down. Furthermore, evidence for a calcium-binding factor in chick intestinal mucosa indicates that it is a protein (Wasserman and Taylor, 1966). Using radioactive proline, a stable protein was found associated with the cell wall of plants (Sadava and Chrispeels, 1969). While the labelling was done on non-calcifying (carrot) tissue, the association of protein with calcification seemed worthwhile for investigation if protein could be demonstrated in the coralline cell walls also.

Phosphatase and carbonic anhydrase have been found in locations of calcification. Phosphatase has been suggested as hydrolysing phosphate groups (Simkiss, 1964). The phosphate groups inhibit crystal formation by settling on a crystal surface and interfering with continued growth of the crystal lattice. Alkaline phosphatase has been found during the formation of calcareous skeletons (Simkiss, 1964), in bone (Nichols, 1967) and in crustacean cuticle (Waterman, 1960). Cartilage and alkaline phosphatase formation was induced
in decalcified tooth (dentin) matrix when implanted in rat abdominal muscle (Huggins and Urist, 1970). Acid extracted matrix contained no alkaline phosphatase, but within 24 hours, the enzyme was rising steeply in the implant but not in the muscle.

Carbonic anhydrase, which catalyses the reaction

\[ \text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3 \]

also increases the conversion rate of carbon dioxide to bicarbonate ions in aqueous solutions in plants and animals (Enns, 1967). Coral with zooxanthellae calcifies faster in the light than in darkness. Without zooxanthellae, coral has a low calcification rate, independent of light intensity (Goreau, 1961). Since carbonic anhydrase is found in zooxanthellae, the involvement of that enzyme in calcification is suggested. Goreau has postulated calcification proceeding in the following manner:

\[ \text{Ca}^{++} \rightarrow \text{organic matrix} \rightarrow \text{bicarbonate} \rightarrow \text{carbonic acid + carbonate salt} \leftarrow \text{bicarbonate salt} \]

Thus, calcium carbonate precipitation is controlled by the removal of carbonic acid by carbonic anhydrase, producing either more bicarbonate to initiate further mineralization or more carbon dioxide for the zooxanthellae. Carbonic anhydrase has been shown to be present in fly larvae which form a partition, partly calcium carbonate, in the shells of their snail prey (Knutson, Berg and Edwards, 1967), and in the calciferous glands in the earthworm which are associated with calcium elimination through calcite production (Crang, Holsen and Hitt, 1968).

Radiosulphur-35 was used to investigate the pathway of sulphur in the coralline algae. Thalli on cover slips present a wide range
of maturity (calcification) on a monostromatic basis, simplifying resolution of the radioisotopic label. Location of sulphur in the corallines would provide useful information on sulphation of carbohydrates in relation to calcification. Because glucose, mannose and xylose had been found to be present in the cell walls of red algae (Cronshaw, Meyers and Preston, 1958; Putman, 1965), polyxylan in the green alga *Eryopsis* (Frey-Wyssling and Muhlethaler, 1965) and glucomannan in the green alga *Hydrodictyon* (Frey-Wyssling and Muhlethaler, 1965), determination of the index of refraction of the decalcified coralline cell wall was carried out to possibly identify these or other polysaccharides. Molecular configuration is the important foundation for calcification, and it was shown by histochemical means in this investigation that there are changes in the coralline thallus in regions of differing maturity and progression of calcification. Birefringence of the decalcified thallus especially informative, demonstrating the ordered structure seemingly necessary to provide nucleation sites for oriented crystals. The study of birefringence in the coralline algae led to the disclosure that calcite alone was not responsible for the results. Preliminary use of polarizing lenses had been done to determine whether the crystal polymorph was aragonite or calcite. Birefringence occurred along the cell wall region to the thallus margin, suggesting the presence of calcite crystals along the entire intercellular area (Figure 81 A). However, when sections for electron microscopy showed no sign of calcification near the coralline periphery (Figure 59), it became necessary to investigate the cause of birefringence.
Birefringence is the result of a difference in the index of refraction for light vibrating parallel to a fibre and perpendicular to a fibre (Bunn, 1953). The refractive index is dependent on the direction of the electrical disturbance caused by electromagnetic waves, not the direction that the light is traveling (Bunn, 1953). Refractive indices are determined by the Becke line. Fibres are immersed in various liquids under polarizing lenses, and the Becke line (bright line) moves toward the medium of higher refractive index as focus is raised (Mauersberger, 1954; Chamot and Mason, 1958).

Birefringence can occur as form or intrinsic birefringence, both due to morphological properties (Frey-Wyssling, 1953). Double refraction (form birefringence) is measured by \( n_{II} - n_I = \) retardation of light perpendicular to the fibre. Form birefringence is due to special shape and orientation, with long molecules or rodlet texture producing positive birefringence and platelet or layer texture producing negative birefringence (Chamot and Mason, 1958; Frey-Wyssling and Mühlethaler, 1965). The mounting medium penetrates the object and alters its optics by imbibition, and when the index of refraction of the liquid equals that of the structure, birefringence disappears (Frey-Wyssling and Mühlethaler, 1965). The chain molecules are themselves anisotropic (birefringent) in intrinsic birefringence, so the birefringence of the structure cannot be reduced to zero by changing the index of refraction of the mounting liquid. Birefringence increases with increased orientation of the molecules.

Birefringence has been used as an indirect method to investigate submicroscopic morphology, to be confirmed later by electron microscopy (Frey-Wyssling, 1957). Cell wall structure has been studied in this
way, showing cellulose to be birefringent and pectin to be amorphous and not birefringent (Frey-Wyssling and Mühlethaler, 1965). All the hemicelluloses are amorphous carbohydrates which separate into hexoses (glucose, mannose), pentoses (xylose) and uronic acids (glucuronic, galacturonic) upon hydrolysis. Histochemically, pectic material cannot be distinguished from uronides (Frey-Wyssling and Mühlethaler, 1965).

Xylan, mannan and glucan are known to occur in fibrillar form (Frey-Wyssling and Mühlethaler, 1965). Crystallization depends on a regular structure to form a chain lattice, usually 1,4-linkages (instead of 1,3-linkages which produce branching and helical structure), and hydration of such a level as to allow crystallization in an aqueous medium. Finding an index of refraction in the coralline organic matrix similar to that of one of the sugars would be indirect evidence for that compound being present.

Results

Histochemistry

For localization of specific compounds or active sites, staining was done on frozen sections as well as thalli grown on cover slips. The histochemical reactions were identical on the two types of tissue unless a distinction is made when commenting on the results. Cover slips provided a convenient way to stain several coralline thalli simultaneously and to provide controls for the staining technique by the reaction of other algae (green and brown) and organisms present on the cover slip. With the exception of the acetone-Sudan Black B which is discussed in lipid staining, the corallines, calcified and
decalcified, did not take up the dyes readily and maximum staining times were used in all cases.

**Polysaccharides.** The results of histochemical tests for polysaccharides are given in Table 4. It can be seen in the table that no conclusive positive reaction resulted for either pectin or cellulose in cover slip thalli. Ruthenium red stained a band 5 cells wide and 3-5 cells from the thallus periphery in the frozen sections, but no control was prepared. In repeated tests, no staining occurred in the reaction or control thalli using the ruthenium red or iodine-lithium chloride for cellulose. The green algae, with cellulose cell walls (Fritsch, 1959), always gave a positive reaction when iodine-lithium chloride was used to stain cover slips.

The results with alcian blue, at pH 0.5 to stain the sulphated polysaccharides are shown in Figure 67A. It can be seen in this figure that the cover cells and intercellular areas reacted positively. In contrast, the reaction with alcian yellow for acid polysaccharides was inconclusive. The reaction of the cover cells appeared slightly deeper yellow than the control (Figures 67B and 67C), but the dense lamellae of the cover cells may have caused a color concentration. In frozen sections, a band of cells approximately 5 cells from the thallus periphery stained blue.

The PAS technique (Figure 68) demonstrated carbohydrates in the thalli, with a stronger reaction in the decalcified thallus. The stain reacts with end groups such as

\[
\begin{align*}
\text{C - OH} & \quad \text{or} \quad \text{C - OH} \\
\mid & \quad \text{or} \quad \mid \\
\text{C - OH} & \quad \text{C - NH}_2,
\end{align*}
\]
## Table 4

### Histochemical stains and results for Polysaccharides

<table>
<thead>
<tr>
<th>Stain</th>
<th>Result</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruthenium red</td>
<td>+ pectin,</td>
<td>- hemi-cellulose</td>
</tr>
<tr>
<td>(Davenport, 1960 after</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Little and Mackey, 1953)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Pearse, 1960)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iodine-lithium chloride</td>
<td>- cellulose</td>
<td></td>
</tr>
<tr>
<td>(Davenport, 1960 after</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post and Laudermilk, 1942)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcian blue and yellow</td>
<td>+ sulphated</td>
<td>± acid polysaccharides</td>
</tr>
<tr>
<td>(Parker and Diboll, 1966)</td>
<td>polysaccharides</td>
<td></td>
</tr>
<tr>
<td>Periodic acid/Schiff (PAS)</td>
<td>+ carbohydrates</td>
<td></td>
</tr>
<tr>
<td>(Pearse, 1960 after</td>
<td></td>
<td></td>
</tr>
<tr>
<td>McMannus)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bi-Col method</td>
<td>± acid mucopolysaccharides</td>
<td></td>
</tr>
<tr>
<td>(Pearse, 1960 after</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wolman, 1956)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylene blue</td>
<td>+ sulphate</td>
<td></td>
</tr>
<tr>
<td>(Pearse, 1960 after</td>
<td>groups</td>
<td></td>
</tr>
<tr>
<td>Dempsey and Singer)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toluidine blue</td>
<td></td>
<td>+ and metachromasia</td>
</tr>
<tr>
<td>(Pearse, 1960 after</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kramer and Windrum)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**

- a Frozen sections.
- b Control: incubation with pectinase.
- c Controls: methylation; methylation followed by saponification.
- d Controls: acetylation; acetylation followed by saponification.
- e Control: no incubation with acid ferrocyanide.
Figure 67

Results of staining with alcian blue (pH 0.5) and alcian yellow for sulphated and acid polysaccharides, respectively, are shown in (A), after removal of the sulphide (B) and after removal of the sulphide and carboxyl groups (C). The cover cells and intercellular area indicate the presence of a sulphated polysaccharide, and (B) faintly suggests there may be acid polysaccharides in the cover cells. Cover cell open arrow; intercellular area, thin arrow. X 1,200.
Figure 68
Periodic acid/Schiff staining for carbohydrates is shown on an untreated coralline (A) and on a decalcified, acetylated (control) coralline (B). The control thallus (B) stains lighter and less evenly than the reaction thallus (A). X 200.

Figure 69
The Bi-Col method demonstrates a weak acid mucopolysaccharide reaction (arrow) in the coralline thallus. X 500.

Figure 70
The staining of corallines with methylene blue at pH 2.62 is shown in (A) and at pH 4.66, the extinction point, in (B). X 500.
but a positive reaction (without other support) is not conclusive evidence of polysaccharides. For confirmation, the decalcified, acetylated control (Figure 68B) showed less reaction than the calcified thallus (Figure 68A), despite heavy PAS positive tests on the decalcified thalli.

The Bi-Col reaction was very weakly positive (Figure 69). However, in contrast, no reaction was detected in the control. Sulphated hyaluronic acid and compounds containing free sulphuric or phosphoric radicals are stained blue by the method (Pearse, 1960).

Methylene blue was used at pH 2.62, 3.62, 4.66, 5.32, 6.12, 6.99, 7.42, and 8.18. Positive staining occurred at all points except pH 4.66, the extinction point (Figure 70A and 70B). Staining below pH 4 indicates sulphate groups (Baker, 1958).

The results of toluidine blue staining is shown in Figure 71. The thallus (Figure 71A) demonstrates the changes in metachromasia in different thallus areas. The calcified region is visible by the scattered open areas and magenta stain. The thallus edge did not stain in the calcified thalli, but a metachromatic band is seen approximately 5 cells wide and 3 cells from the periphery in both calcified and decalcified algae. Toluidine blue is a basic dye and stains most basophils purple or red (Baker, 1958). Some of the more common basophils that are chromotropic are sulphuric, phosphoric and carboxyl groups and sulphuric esters of polysaccharides. It is seen in Figure 71B and 71C that decalcification allows staining of the cells below the deep blue cover cells.
Figure 71

Metachromasia resulting from toluidine blue staining is shown in (A). The more extensively stained decalcified thallus (B) suggests that toluidine blue penetrates to these cells more readily than in the calcified thallus (C) where only the cover cells and submarginal band stain. Arrows indicate the submarginal band of cells which reacts more strongly to staining. A, X 100. B, X 700. C, X 500.
**Sulphydryl and sulphide groups.** The results of staining for S-H and S-S bonds are given in Table 5. The neotetrazolium stain reacted weakly in the frozen and cover slip thalli. In the frozen sections, a band approximately 5 cells wide and 5 cells from the thallus edge indicated the presence of sulphur bonds. In the cover slip thalli, only the cover cells stained. The ferricyanide stain clearly demonstrated the occurrence of numerous sulphide and a few sulphydryl groups by the blue color (Figure 72). The terminal cells show less coloration than the interior of the thallus.

**Proteins.** The association of collagen with crystal nucleation in vertebrates suggested a possible parallel in the corallines, and hence, histochemical tests for protein were performed. Positive results with all techniques were recorded (Table 6), but these may be due to the use of specific animal stains on algae and not an indication of protein. All controls using deamination gave negative reactions and tend to support positive identification of cell wall protein. Figures 73 and 75 for reactions with aniline blue collagen and mercury-bromphenol blue, respectively, indicate reaction with protein by the blue color. The peripheral cells stained intensely blue, but the color was difficult for photo reproduction. However, in contrast, no blue color was visible in the controls.

In the ninhydrin-Schiff method the production of a pink or magenta color in the tissue is an indication of the presence of proteins. The decalcified corallines stained more deeply than the calcified corallines did (Figure 74).
Table 5
Histochemical Stains and Results for Sulphides and Sulphydryls

<table>
<thead>
<tr>
<th>Stain</th>
<th>Result</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neotetrazolium (Casselman, 1959</td>
<td>+ S-S</td>
<td>S-S groups</td>
</tr>
<tr>
<td></td>
<td>- S-H</td>
<td>groups</td>
</tr>
<tr>
<td>after Gomori, 1956)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferric ferricyanide (Pearse, 1960</td>
<td>÷ S-S</td>
<td>S-S and S-H groups</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>after Chevremont and Frederic, 1943)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Controls: S-S converted to S-H; S-H group blocked.

Table 6
Histochemical Stains and Results for Proteins

<table>
<thead>
<tr>
<th>Stain</th>
<th>Result</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aniline blue collagen (Davenport, 1960</td>
<td>+</td>
<td>connective tissue (collagenous), ground</td>
</tr>
<tr>
<td></td>
<td></td>
<td>substance (cartilage, etc.)</td>
</tr>
<tr>
<td>(after Mallory, 1938)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mercury-bromphenol blue (Pearse, 1960</td>
<td>+</td>
<td>proteins</td>
</tr>
<tr>
<td>(after Bonhag)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minhydrin-Schiff (Pearse, 1960 after</td>
<td>+</td>
<td>protein-bound NH₂</td>
</tr>
<tr>
<td>Yasuma and Itchikawa, 1953)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milligan's trichrome (Humason, 1967</td>
<td>+</td>
<td>nuclei, muscle and collagen</td>
</tr>
<tr>
<td>(after Milligan, 1946)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Control: deamination.
Figure 72

The results of staining with ferric ferricyanide for sulphydryl and sulphide groups are shown for coralline thalli where S-S is converted to S-H (A); where no conversion occurred and only S-H groups stained (B); and where the S-H groups were blocked (C). The deep coloration in (A), and much lighter in (B), suggests the occurrence of predominately S-S groups and only a few S-H groups. A, B, X 200. C, X 500.
Figure 73

Aniline blue collagen indicates proteins by a blue color, seen in the peripheral region of the coralline thallus. X 300.

Figure 74.

A magenta color indicates protein in the ninhydrin-Schiff method. A slightly deeper color occurred in the calcified reaction thallus (A), especially in the cover cells, than in the decalcified coralline control thallus (B).

A, X 300. B, X 1,000.
Figure 75

A deep blue coloration indicating protein occurred at the coralline thallus periphery with the mercury-bromphenol blue staining (arrow). X 200.

Figure 76

Milligan's trichrome technique produced quite different results in a decalcified coralline (A) and a calcified coralline (B). Calcite may take up the green stain, a possible explanation for the green proximal areas in (B). X 700.
Staining reactions with Milligan's trichrome technique are presented in Figure 76A and 76B. Magenta and green indicate protein and collagen, respectively. The unusual results in the calcified thallus (Figure 76B) may be due to calcite uptake of the fast green after prior exposure to acids.

**Lipids.** The reaction to the acetone-Sudan Black B method for lipids (Table 7) was general throughout the calcified and decalcified coralline thalli. Cover slip algae were stained for 18 hours for optimum results. It was found in these preparations that the chromatoplasts stained black.

**Calcium.** A stain that is claimed to produce very specific results in bone was used to determine calcium in the coralline algae (Table 7). Glyoxal bis (2-hydroxyanil) stained the entire reaction thalli deep red (Figure 77A). The cell wall is stained in green algae with scattered dots of red. In contrast, the coralline controls were completely devoid of any red color (Figure 77B).

**Enzymes.** Table 8 presents results for specific histochemical reactions for carbonic anhydrase, alkaline phosphatase, and acid phosphatase. It can be seen in Table 8 that negative results were obtained using the test for alkaline phosphatase. This is expected, because alkaline phosphatase is rarely found in plants. Reaction for acid phosphatase was positive in light microscopy as is shown in Figure 78. When tests were made for acid phosphatase in electron microscopy (Figure 79), they were inconclusive, mainly due to the poor condition of the tissue after incubation treatment. The control sections appeared the same as the active enzyme sections, showing a
precipitate in the chloroplasts along the lamellae. The mitochondria were too badly broken to positively identify, but the organelles appeared to be stained in both control and reaction sections. Carbonic anhydrase reaction was weak in the frozen sections, and the reaction was difficult to interpret in cover slip thalli (Table 8). Staining occurred on all cover slips except those incubated without ammonium sulphide. Apparently Diamox (acetazolamide, sodium salt) did not inhibit carbonic anhydrase in the coralline algae. The stain was heavy throughout the thalli, especially in the marginal 20 cells and cover cells. However, until inhibition of the enzyme can be accomplished or proven, the results must remain inconclusive.

Sulphur uptake

The pathway of sulphur into coralline algae was followed by pulse-labelling of cover slip thalli with radiosulphur-35. Cover slips were placed in the ocean for four weeks to allow settling of the corallines, then brought into the laboratory for experiments. Three light conditions were used to determine effects produced by photosynthesis. After pre-conditioning in continuous dark, continuous light and normal light for a day, radiosulphur-35 was added to the sea water. The results are summarized in Table 9 and Figure 80. The dark incubated thalli incorporated very little sulphur, and autographs indicated only scattered label along cell edges after 4 hours incubation. Radioautographs from preparations in continuous light and normal daylight thalli demonstrated concentration of sulphur in the cover cells. The appearance of the label was apparent after 0.5 hours in continuous light and after 4 hours under normal daylight
Table 7

Histochemical Stains and Results for Lipid and Calcium

<table>
<thead>
<tr>
<th>Stain</th>
<th>Result</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone-Sudan Black B (Pearse, 1960 after Berenbaum)</td>
<td>+</td>
<td>lipids</td>
</tr>
<tr>
<td>Glyoxal bis (2-hydroxyanil) (Kashiwa, 1966)\textsuperscript{a}</td>
<td>+</td>
<td>calcium</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Controls: decalcification; decalcification and incubation with Ca-G\textsubscript{2}HA granules; decalcification and incubation in artificial sea water with CaCl\textsubscript{2} added.

Table 8

Histochemical Stains and Results for Enzymes

<table>
<thead>
<tr>
<th>Stain</th>
<th>Result</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase (Pearse, 1960 after Fredricson, 1952)\textsuperscript{a}</td>
<td>-</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>Acid phosphatase (Pearse, 1960) after Gomori, 1950\textsuperscript{b}</td>
<td>+</td>
<td>acid phosphatase</td>
</tr>
<tr>
<td>(Parka and Anderson, 1967)\textsuperscript{bc}</td>
<td>±</td>
<td>acid phosphatase</td>
</tr>
<tr>
<td>Carbonic anhydrase (Pearse, 1960: Hausler, 1958 after Kurata)\textsuperscript{d}</td>
<td>±</td>
<td>carbonic anhydrase</td>
</tr>
<tr>
<td>(Davenport, 1960)\textsuperscript{e}</td>
<td>±</td>
<td>carbonic anhydrase</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Control: incubation without substrate
\textsuperscript{b} Controls: incubation with NaF; incubation without substrate.
\textsuperscript{c} Stained for electron microscopy
\textsuperscript{d} Controls: incubation with Diamox; incubation without substrate.
\textsuperscript{e} Frozen sections.
Figure 77
Staining for calcium with GBHA resulted in an intense red color over the entire coralline thallus (B). After decalcification with EDTA, no red coloration was visible, (A). A, X 1,200. B, X 500.

Figure 78
Acid phosphatase was located throughout the coralline, with the submarginal band as a peripheral boundary. X 500.
Acid phosphatase staining for electron microscopy produced the same appearance in both reaction and control sections, showing a precipitate along the chromatoplast lamellae. Chromatoplasts, cp; mitochondria, m; cell wall, cw. Glutaraldehyde-Pb (OH)$_2$. X 21,500
<table>
<thead>
<tr>
<th>Hours in 358</th>
<th>Incubation Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal Daylight</td>
</tr>
<tr>
<td>0.5</td>
<td>light general label</td>
</tr>
<tr>
<td>1</td>
<td>light label in cover cells</td>
</tr>
<tr>
<td></td>
<td>moderate-heavy label in cover cells</td>
</tr>
<tr>
<td>2</td>
<td>light label in cover cells</td>
</tr>
<tr>
<td></td>
<td>moderate-light label in thallus center</td>
</tr>
<tr>
<td>4</td>
<td>moderate label in cover cells</td>
</tr>
<tr>
<td></td>
<td>moderate label at lateral edges of branches where anastomosing</td>
</tr>
<tr>
<td></td>
<td>no label within thallus</td>
</tr>
</tbody>
</table>
Figure 80

Radioautographs of coralline thalli after incubation with $^{35}$S for 2 hours. Arrows point out the areas of label concentration.

(A) normal daylight - scattered light label
(B) continuous dark - heavy label in cover cells
(C) continuous light - heavy label in cover cells and moderate label throughout

$\times 650$. 
conditions. After 4 hours, the light conditioned thalli were labelled strongly in the 3 peripheral cells of the thalli, as well as in cover cells and the fronts of cells up to 0.7 mm from the algal edge. Normal daylight thalli showed incorporation of the isotope into cover cells and at the lateral edges of scattered cells after 4 hours.

**Birefringence and index of refraction**

Coralline algae grown on cover slips, left in the ocean for four weeks for algal settling, were observed for birefringence. Before decalcification, birefringence occurred in walls parallel and perpendicular to the direction of growth (Figure 81A), with extinction points every 90°. After decalcification with neutral EDTA, birefringence was fainter and occurred only in a direction parallel to the cell wall fibres (Figure 81B). Decalcification was verified using GEL stain (Kashiwa, 1966). Decalcification with dilute or concentrated HCl apparently disrupted the fibre organization and birefringence was extinguished. Birefringence of calcified thalli was not affected by concentrated NaOH nor by overnight incubation in a 1% trypsin (CalBiochem, 3100 NF trypsin units/mg and 10 NF chymotrypsin units/mg) solution, pH 7.75, at room temperature.

To ascertain if the birefringence of decalcified coralline algae was form or intrinsic, decalcified geological thin sections and cover slip thalli were examined in refractive index oils. The index of refraction was also used to indicate possible matrix constituents. Refractive index oils from 1.470 to 1.590 at intervals of 0.01 were allowed to penetrate decalcified geological thin sections and cover slip thalli overnight before examination with polarizing lenses in a
Figure 81

Photomicrographs of coralline thalli showing birefringence present at 90°, or any angle (arrow) in the calcified coralline thallus (A) and occurring only in one direction (arrow) in the decalcified thallus (B). X 200.
Zeiss Universal microscope. For a more accurate determination of the refractive index, refractive index oils were added at intervals of 0.002 between 1.512 and 1.528 and examined in a Zeiss phase contrast microscope. The results are presented in Figure 82. Because all birefringence was extinguished at 1.518, the refractive index of decalcified corallines is equal to that value and the identification of form birefringence was made.

Some materials and their refractive indices are listed in Table 10 for comparison with the value obtained for decalcified coralline algae.

Table 10
Refractive Indices of some Materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Refractive Indices</th>
</tr>
</thead>
<tbody>
<tr>
<td>calcium carbonate</td>
<td>1.530, 1.681, 1.685</td>
</tr>
<tr>
<td>aragonite</td>
<td>1.658, 1.486</td>
</tr>
<tr>
<td>calcite</td>
<td>1.53</td>
</tr>
<tr>
<td>starch (amylose)</td>
<td>1.53</td>
</tr>
<tr>
<td>cellulose</td>
<td>1.594, 1.532</td>
</tr>
<tr>
<td>flax</td>
<td>1.596, 1.528</td>
</tr>
<tr>
<td>ramie</td>
<td>1.578, 1.538</td>
</tr>
<tr>
<td>cotton</td>
<td>1.591, 1.538</td>
</tr>
<tr>
<td>silk</td>
<td>1.556, 1.547</td>
</tr>
<tr>
<td>wool</td>
<td>1.545+, 1.545</td>
</tr>
<tr>
<td>soybean</td>
<td>1.536, 1.532</td>
</tr>
<tr>
<td>zein (vegetable protein)</td>
<td>1.490, 1.505, 1.620</td>
</tr>
<tr>
<td>chitin</td>
<td>1.517, 1.544, 1.546</td>
</tr>
<tr>
<td>xylose</td>
<td>1.3349 - 1.3620</td>
</tr>
<tr>
<td>decalcified coralline</td>
<td>1.518</td>
</tr>
</tbody>
</table>
Figure 82

Curve of form birefringence of decalcified coralline algae.
Discussion

Although the histochemical tests employed did not provide evidence for the actual chemical composition of the coralline cell wall, the analyses gave fruitful information about calcification. Of special interest is the sub-marginal band of approximately 5 cells wide and occurring 3-5 cells from the thallus periphery. In nearly all of the techniques employed, the strongest reaction took place in this region. The obvious implication is that the area is one of changing structure. This seems a reasonable assumption, because if only active growth was responsible, it would follow that the reaction would be the same at the coralline periphery.

The toluidine blue technique (Figure 71A) strikingly demonstrates the different functional regions of the thallus by the marked change in metachromasia, i.e., the magenta colored sub-marginal band of cells. Metachromasia is a hypsochromatic effect, a shift to longer wave length: blue to violet to red. Two factors affect the degree of metachromasia, the nature of the acid groups and the degree of separation in space (Baker, 1958). Sulphuric esters of polysaccharides are common chromotropes, and polysaccharide sulphates are common structural units in the red algae (Anderson, Dolan and Rees, 1965). Further evidence for the presence of sulphur in the sub-marginal band of cells comes from neotetrazolium staining on frozen sections, and ferric ferricyanide localization of S-S groups in sub-terminal cells and the thallus interior. Alcian blue, specific for sulphated polysaccharides, stained a blue band in the frozen sections.

The hydrophilic quality of sulphated polysaccharides may be a factor accounting for the intense staining capacity of the sub-
marginal band of cells. Water soluble dyes would be expected to be concentrated in such an area, accounting for positive results to many diverse stains. Free sulphides were implicated in early calcification by Schiffman, Martin and Miller (1970). Cations such as iron were bound via S-H. If p-mercuribenzoate (pMB), specific for S-H, was added, mineralization was prevented. This suggests cellular control, not mineral-matrix reaction alone. The basophilic qualities may also result in a proton affinity important to calcite nucleation. Johnson (1960) found histologic changes in the turkey leg tendon undergoing mineralization that parallels those suggested for coralline algae. In the tendon, the polysaccharide developed metachromasia prior to mineralization. Subsequently, loss of metachromasia and decreased PAS stain occurred with ensuing calcification.

Uptake of sulphur was influenced by light conditions and, thus, implication of energy through photosynthesis. The concentration of sulphur in the peripheral cells in the continually illuminated thalli is not contradictory to the proposed sulphated polysaccharide sub-marginal band of cells. The sulphur appears to follow metabolic activity, first appearing in the cover cells with dense lamellae (photosynthetic?). The marginal cells are actively dividing and would receive energy and materials such as sulphates from the synthesizing cover cells. Sulphur is also found where branches are anastomosing, suggesting that sulphur may be transported to areas where no further growth will occur and preparation for calcification has begun. In time, sulphur may be reduced and incorporated into S-amino acids and linked to polysaccharides. Fritsch (1959) states
that calcification is more dense in well-illuminated individuals. If sulphonation precedes calcification, the amount of sulphur may be directly proportional to the amount of light.

Methylene blue stain provides information on the iso-electric point of tissue. The dye enters the cell as the non-ionic leucobase (colorless form, Baker, 1953). Because the dye is ionized in solution, it is reduced in the vicinity of the cell and reoxidized in the cell to restore the chromophore. Proteins may oxidize when on the alkaline side of the iso-electric point (Harris and Peters, 1953), thus, staining at pH 2.62 indicates strong basophilic properties. Be and Ericson (1963) suggest that the qualities of a sulphated mucopolysaccharide which is strongly basophilic would present a potentially calcifiable matrix by providing an initial deposition site for calcium.

Many mucopolysaccharides are in the form of carbohydrate-protein complexes, making the presence of protein in the coralline cell wall not unexpected. The verification of protein in the carrot tissue by Sadava and Chrispeels (1969) further supports the histochemical finding of protein. A portion of chondroitin sulphate is bound to protein (Fruton and Simmonds, 1959), and in egg whites, protein is bound to a neutral mucopolysaccharide, containing hexosamine and other sugar residues but no glucuronic acid or sulphate (Fruton and Simmonds, 1959). The absence of glucuronic acid would explain the negative results obtained with stains for hemi-cellulose. The bonds between the carbohydrate and protein include calcium and sulphur (Kwart and Shashoua, 1957). The bonding is:
Thus, while the positive results for protein may be the result of anomalous staining or due to hydrophilic or structural qualities of the tissue, there is data to suggest that proteins are present and available to react.

Form birefringence of decalcified coralline thalli (Figure 82) provides evidence for the presence of ordered structure in the cell wall. The polysaccharides xylose, mannose, glucose and galactose can all occur in fibrillar (birefringent) form, while pectin is an amorphous material. The interpretation of the index of refraction encounters many difficulties in an heterogenous system. The refractive index of 1.518 (Figure 82) obtained for the corallines suggests the presence of xylose (I.R. = 1.517, Table 10), but saccharide combinations and associated proteins will influence the index of refraction. Combinations will usually present the average index of refraction, and proteins generally raise the value. Thus, while pectin and cellulose may occur in minor amounts, the index of refraction would be nearer 1.53 if the cell walls consisted of cellulose units.

Carbonic anhydrase and acid phosphatase were demonstrated in coralline algae by histochemical tests (Table 8). However, the enzymes could not be linked to specific sites or times of calcification. Acid phosphatase occurred throughout the thallus with the exception of peripheral cells (Figure 78). This distribution at intercellular areas suggests the enzyme is present in calcifying
regions, and that structural changes occurring in the sub-marginal band of cells are necessary for the manifestation of acid phosphatase activity. Carbonic anhydrase, more generally distributed in the thallus, seemed to be associated with areas of high metabolic activity. The involvement of the enzyme with CO₂ fixation in photosynthesis would tend to support the localization in the tests as due to general physiology and not influenced by calcification processes. Enzyme involvement is important to an explanation of mineral regulation. Suggestions for enzyme correlation with calcification were seen in the results of histochemical localization of acid phosphatase. Ultrastructural localization would be valuable to investigate nucleation-enzyme relationships. Studies on energy requirements and temperature optima would also provide information on the role of enzymes in calcification.
CHAPTER V
The Structure and Calcium Metabolism
of the Organic Matrix

Introduction

The relationship of the organic matrix to inorganic crystals in biological calcification is an extremely close and important association. The organic matrix appears to be intimately involved with crystal nucleation, formation and orientation in calcifying tissues (Gregoire, 1957; Glimcher, 1958; Glimcher, 1960; Johnson, 1960; Mergenhagen, Martin, Rizzo, Wright and Scott, 1960; Watabe and Wilbur, 1961; Rizzo, Martin, Scott and Mergenhagen, 1962; Piez, 1963; Wilbur and Watabe, 1963; Watabe, 1965; Mahlethaler, 1967; Towe and Hamilton, 1968; Schiffman, Martin and Miller, 1970; Travis, 1970). Certain basic parallels seem to be present in calcifying plants and animals, foremost of which is the similarity between the matrices and fibrous elements. The understanding of calcification mechanisms in multicellular animals will be made easier by studies that deal with simpler systems, and with that concept in mind, this investigation of the organic matrix was begun using coralline algae.

The organic matrices involved in mineralization of diverse organisms have an underlying unity in possessing the following qualities (Travis, 1970): 1) Large amounts of mineral can be deposited within an ordered matrix without disruption of the matrix organization. 2) The matrix influences the size and orientation of the mineral. 3) Complete mineralization of the matrix does not occur, at least in the early stages of maturation. 4) There are
changes in composition and structure of the matrix as maturation and mineralization takes place. These criteria are all fulfilled by the coralline matrix, as pointed out in earlier chapters.

Matrices for calcification are shown to be composed of fibres, sheaths and membranes. Fibres are the most commonly occurring, especially among the vertebrates, and form extensive fibrous sheets. Collagen fibres are found in bone, dentin, echinoderms, and older sponge spicules (Schiffman, Martin and Miller, 1970). Membranes are involved in calcification in plants and invertebrate tests. Coccoliths appear to be formed between two membranes which move apart as the crystals are deposited (Wilbur and Watabe, 1963). In the leave of Lemma, membranes occur in pairs with crystals occurring between in linear progression (Arnott and Pautard, 1970). Sheaths are present in enamel and young sponge spicules (Schiffman, Martin and Miller, 1970).

Mineralization within a matrix is an ordered process, and where deposition occurs randomly, the crystals may become oriented if they grow in the developing matrix. If there is no association with a matrix, minerals grow without morphological or crystallographic orientation (Travis, 1970). Recrystallization may cause the growth of large crystals at the expense of the smaller ones.

Nucleation of crystals and their growth is discussed at length by Glimcher (1958, 1960). He suggests that the positions of certain reactive groups create specific regions which act as nucleation sites. That is, the stereochemistry of the major organic components is the main factor in mineralization. Glimcher cites as an example
that any alteration in the axial repeat of native collagen \( (640\AA) \) effects loss of the ability to calcify. Yet, the ordered fibrous protein paramyosin is unable to calcify, illustrating the necessity of specific reactive groups as well as periodicity. Collagen shows nucleation centers at every axial repeat, in the dense band areas where the most active sites for electron stains and amino acids with reactive chain groups are found (Glimcher, 1958). Reactive groups may contain anions that complex with calcium ions initially, or amino acids may have spatial arrangement for selective binding of calcium or carbonate. Or reactive groups may be arranged sterically to approximate crystal size, needing only the addition of ions to establish nucleation of a crystal.

Normal nucleation appears to involve more than just a physical precipitation on the organic matrix. When the rat tail tendon was removed and implanted into the peritoneal cavity, calcification occurred in the transplant before it did in normal rat tails (Mergenhagen, Martin, Rizzo, Wright, and Scott, 1960). In the turkey tendon, the original collagen bundles were transformed to osteoid material by adding organic matrix material before mineralization developed (Johnson, 1960). An extract from *Bacterionema matruchotii* was prepared by dialysis that appeared to contain a factor responsible for calcification (Ennever, 1963). When the factor was added to a solution containing calcium and phosphate, precipitation of hydroxyapatite took place. Wilbur and Watabe (1963) demonstrated that the crystal polymorph formed from calcium bicarbonate solutions depended on the suspended molluscan organic matrix (aragonitic or calcitic). Non-viable bacteria mineralized at a faster rate than viable bacteria
when both were implanted in rat peritoneal cavities (Rizzo, Martin, Scott and Mergenhagen, 1962), suggesting the presence of preventive as well as inductive processes for calcification.

The matrix of the coralline algae was studied to determine structure, periodicity and biological factors involved in calcite deposition. Electron microscopy was employed to resolve fibre arrangement in the cell wall and the existence of periodicity within fibres or complexes. The incorporation of calcium by the matrix was followed by using radiocalcium in media with live coralline thalli, decalcified sections and thalli, and with decalcified matrix slurries.

Results

Calcium uptake

Geological thin sections. After decalcification in neutral EDTA, the slides with geological thin sections affixed were suspended in calcium-free Gold's artificial sea water with 10 uCi/liter of $^{45}$CaCl$_2$ added. The incubating medium was stirred continuously to assure equal distribution of the radiocalcium. Samples were removed and monitored with a portable Geiger-Müller counter held approximately 0.5 cm from the sample. The results are presented in Figure 83. Control sections were calcified, left for the maximum time to determine calcium exchange.

Decalcified coralline thalli. Coralline algae were grown on cover slips left in the ocean for four weeks to allow algae to settle. The cover slip corallines were decalcified in neutral EDTA for 19 hours. Warshawsky and Moore (1965), working with bone, found enzymes still active after decalcification in neutral EDTA at $4^\circ$C, and for this
Figure 83

Calcium uptake by decalcified geological thin sections incubated in radiocalcium enriched Gold's artificial sea water.
reason, cold decalcification was employed with one half of the corallines. The other half was decalcified at 22°C, then dried at room temperature. All the samples were placed in Gold's artificial sea water at 22°C with 10 uCi/liter of $^{45}$CaCl$_2$ added. The cover slips were removed at periodic intervals and prepared for radioautography. Controls were calcified thalli left for the maximum incubation time. The results are presented in Table 11. The general trend can be seen to be increasing label with increasing time of incubation. However, the 22°C thalli reached maximum labelling by 6 hours, while the 4°C thalli continued to incorporate calcium over the 24 hours that samples were taken.

Table 11
Calcium uptake by Decalcified Coralline Thalli

<table>
<thead>
<tr>
<th>Hours</th>
<th>4°C Thalli</th>
<th>22°C Thalli</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>moderate label over thalli</td>
<td>moderate-heav y label in few scattered areas</td>
</tr>
<tr>
<td></td>
<td>sparse label on cover cells, periphery</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>moderate-heavy label over thalli</td>
<td>scattered, sparse label</td>
</tr>
<tr>
<td></td>
<td>sparse-moderate label on cover cells and periphery</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>same as 2 hours</td>
<td>moderate-sparse scattered label</td>
</tr>
<tr>
<td>12</td>
<td>moderate label concentrated in the mid and peripheral thallus - none inbetween</td>
<td>scattered sparse label</td>
</tr>
<tr>
<td>18</td>
<td>same as 12 hours</td>
<td>scattered, light label</td>
</tr>
<tr>
<td>24</td>
<td>moderate label over all of the thallus</td>
<td>scattered, sparse label</td>
</tr>
</tbody>
</table>

Control: little or no label visible.
Living coralline thalli. Cover slips left in the ocean for 16 days to allow corallines to settle were used to follow calcium uptake. The specimens were kept at 21°C for three days in the laboratory to acclimatize before being placed in sea water with 43.9 uCi/liter of $^{45}$CaCl$_2$ added. The samples were removed and immediately fixed in gluteraldehyde at intervals for radioautography. The results are given in Table 12.

Table 12
Calcium Uptake by Normal Coralline Thalli

<table>
<thead>
<tr>
<th>Hours</th>
<th>Calcium Distribution*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Heavy label on cover cells</td>
</tr>
<tr>
<td></td>
<td>Sparse label at cell edge (outer cell wall?)</td>
</tr>
<tr>
<td>4</td>
<td>Heavy label on cover cells</td>
</tr>
<tr>
<td></td>
<td>Heavy outline at cell edge (more cells than at 2 hours)</td>
</tr>
<tr>
<td></td>
<td>Moderate label in cells, front of cells</td>
</tr>
<tr>
<td>12</td>
<td>Very heavy label on cover cells</td>
</tr>
<tr>
<td></td>
<td>Sparse label at cell edge</td>
</tr>
<tr>
<td></td>
<td>Scattered label intercellularly</td>
</tr>
<tr>
<td>24</td>
<td><em>Porolithon</em> sp.</td>
</tr>
<tr>
<td></td>
<td>Very heavy label on cover cells</td>
</tr>
<tr>
<td></td>
<td>Heavy outline at cell edge</td>
</tr>
<tr>
<td></td>
<td>Sparse-moderate outline at plasmalemma on some cells</td>
</tr>
<tr>
<td></td>
<td><em>Melobesia</em> sp.</td>
</tr>
<tr>
<td></td>
<td>Moderate label over all of the thallus</td>
</tr>
<tr>
<td></td>
<td>Moderate label at plasmalemma, intercellularly</td>
</tr>
<tr>
<td></td>
<td>Sparse label in cover cells</td>
</tr>
</tbody>
</table>

* Most of the labelling occurred in the "band" area, 2-3 cells from the margin of the thallus, unless otherwise mentioned.

All of the data for calcium uptake by coralline thalli were the result of observing several to many thalli on each cover slip. While variation occurred within a group, the overall label distribution was quite consistent.
Electron microscopy

Decalcified coralline tissue. Broken branches of *Porolithon gardineri* were treated with simultaneous 10% formalin fixation and 5% EDTA decalcification (Manning and Butler, 1965), buffered with 1% sodium acetate at pH 5.2, or with gluteraldehyde followed by 5% EDTA decalcification. The *P. gardineri* pieces were treated for 18 hours at 4°C, then transferred to neutral EDTA at room temperature for six weeks to completely decalcify the branches. After thoroughly washing in veronal acetate buffer, the pieces were post-fixed in two ways. 1) The branches were placed in acetone, then ethyl chloride for 1 hour at 6°C (Scott and Nylen, 1959). 2) The branches were placed in 1% OsO₄ in veronal acetate with 1.5% sucrose added for 1 hour at 6°C. Standard dehydration and embedding in Epon 1:1 followed. Figures 84, 85 and 86 show the preparations of the organic matrix.

In Figures 84 and 85, the matrix appears as a sheet of fine fibrils, while the matrix in Figure 86 shows aggregation into separated, thick fibres. The difference in appearance can be explained by the two post-fixation methods employed. This will be discussed later. Evidence for periodicity can be seen along the fibres at approximately 700Å, and larger (400-500Å) condensations or crystal nuclei occur at 700Å intervals on the scattered, short fibres occurring further away from the cell wall proper in Figures 84, 85 and 86. A shorter periodicity of approximately 300Å is found on the fibres in Figure 85 and some of the fibres in Figure 84. The cell wall fibres occupy almost all of the intercellular space in
Decalcified *Porolithon gardineri* showing the extensive organic matrix with periodicities of 250Å, a, and 700Å, b, and crystal nuclei, cn, sometimes separated by approximately 700Å, cn₁. (See Figures 85 and 86 for comparisons.) EDTA/formalin, acetone, ethyl chloride, Pb citrate and Ba(NO₃)₂. X 21,000.
Figure 85

Decalcified *Porolithon gardineri* showing the sheets of fine fibrils with 300Å periodicity, a, and crystal nuclei, cn. (See Figures 84 and 86 for comparisons.) Pit connection, pc. Glutaraldehyde, EDTA, acetone, ethyl chloride, Pb citrate and Ba(NO₃). X 21,000.
Decalcified *Porolithon gardineri* showing the matrix aggregated into thick fibres, probably due to the OsO₄ post-fixation. Crystal nuclei, cn; condensations, C; 700Å periodicity, B. (See Figures 84 and 85 for comparisons.) EDTA/formalin, OsO₄, Pb citrate and Ba₄nO₄. X 21,000.
Figure 85, and the condensations, or crystal nuclei, are much smaller, in the order of 130-200Å. The organic matrix appears as a fine, reticulate network with the suggestion of layers. The fixation method in Figure 85 employs acetone and ethyl chloride but no formalin or OsO₄. The periodicity and condensations appear in all fixation methods. The consistent occurrence of these condensations is indicative that they are not artifacts due to fixation. A deep staining inner cell wall layer has collapsed in the wet freeze-drying method (Figures 84 and 85) and has condensed greatly with the OsO₄ post-fixation (Figure 86). Intracellular organelles have been removed by the decalcification and fixation treatments. No suitable way of decalcifying, then sectioning *Porolithon gardineri* for electron microscopy has been found yet.

**Calcified corallines from cover slips.** In sections of corallines taken near the thallus margin, development of the deep staining layer of the cell wall can be seen (Figure 87). The deep staining layer, occurring outside the plasmalemma, is absent in the newer cells. The layer is approximately 600Å thick at point 4 in Figure 87 and 2,000Å in the mature cell presented in Figure 84. No periodicity is visible in the deep staining layer in the cells in Figure 87, only a thickening of the dark region.

In the heavily calcified sections (Figures 83 and 89), the interaction of matrix and crystal is seen. Double fibrils, approximately 100-150Å wide, can be discerned intimately associated with crystals in Figure 88. These double fibrils have approximately 80Å periodicity. This is demonstrated also in Figure 89. Some crystals have 80Å...
Figure 87

Development of the deep staining (BaMnO₄) cell wall layer in a peripheral section of *Porolithon gardineri*. The thallus periphery is at the right. The stages in formation of the layer are marked 1-4, early to later stages. Nuclei, n; chromatoplasts, cp; pit connections, pc; starch granules, sg; mitochondria, m; central cell vacuole, cv; lateral fusion, lf. BaMnO₄, Pb citrate. X 9,500.
Figure 88

Section of calcified Porolithon gardineri showing the matrix-crystal interaction. Double fibrils of 100Å (thin arrows) and 200Å (open arrows) width are noted, as well as periodicities of 80Å on the fibrils and some crystal edges, z, and 250Å along the cell wall, a. Chromatoplasts, cp; lipid, l; cell wall, cw; pit connection, pc. Glutaraldehyde, OsO₄, Pb citrate and Ba₂InO₄. X 32,000.
Section of calcified *Porolithon gardineri* showing the matrix-crystal interaction. Periodicities of 50-80Å, z, are pointed out on fibres and along the edges of crystal. Double fibrils of 100-150Å (thin arrows) are also marked. Chromatoplast, cp; pit connection, pc. Gluteraldehyde, OsO₄, Pb citrate and BaMnO₄.
periodicity adjoining one or more sides (Figure 88 and 89), suggesting cell wall fibrils that have adhered to the growing crystal.

Shadow cast sections reveal numerous double fibrils of approximately 200Å width in the ground matrix (Figure 90). Some are closely associated with crystals, resembling the double fibrils in Figure 88. The slight discrepancy in size may be due to metal evaporated onto the fibrils or inaccuracy in calculation of the electron microscope magnification. Intercrystallar connections are readily apparent (Figure 90), varying from 100-500 Å wide, and generally occurring at right angles to the cell wall.

Randomly oriented couplets and crystals of approximately 300Å width occur at the edge of a cover cell (Figure 91). There is obvious correlation between crystal and couplet width, and some partially calcified couplets appear to be present. Periodicity is seen along the fibrils making up the couplet at approximately 300Å.

**Calcium uptake by decalcified matrix**

*Porolithon gardineri* branches were brought into the laboratory and carefully cleaned of epiphytic algae and other extraneous matter. After breaking into small pieces, the fragments were ground in a mortar and pestle, followed by final homogenization in a Waring blender with cold decalcifying solution. Approximately 100 ml of the resultant coarse sand was transferred to flasks of 500 ml of neutral EDTA at 4°C which were continuously stirred. Changes of the cold, sterile solution were made weekly for over a month.

Samples were taken in triplicate for each observation, unless otherwise noted, and a control of ⁴⁵Ca-enriched medium without matrix
Figure 90

Shadow cast section of *Porolithon gardineri* showing double fibrils of 200Å (thin arrows) and intercrystallar connections of 100-300Å width (open arrows). Solid arrow indicates shadow angle. Glutaraldehyde, OsO₄, Pb citrate, Pt/Pd shadowed. X 63,000.
Figure 91
Calcified *Porolithon gardineri* showing the random "sheath" and crystal arrangement at the edge of a cover cell. The double fibrils or sheaths have 300Å periodicity (solid arrow). Crystals (thin arrow) are 250-300Å wide and 300Å periodicity can be seen in partially calcified crystals (open arrow). (Acid phosphatase stained.) Glutaraldehyde, Pb(OH)$_2$. X 25,000.
was pipetted through a millipore filter and rinsed with cold Tris buffer, with a final count of 60 ± 10 CPM. Measurements were taken of the pH of the incubating medium before and after incubation of the matrix. Measurements were never recorded above pH 7.0 nor below pH 6.4. Variations in the basic procedure outlined above and in Materials and Methods will be detailed in conjunction with the experiment involved.

Effects of temperature and stirring on calcium uptake by decalcified coralline matrix. After decalcification of the algal sand for one month, groups of decalcified algal cells were seen in microscopic examination along with heavy bacterial contamination. Antibiotics were added to fresh EDTA solution, and, after several rinses of the precipitate, the preparation was left to decalcify for one month longer, with weekly changes of the solution. The antibiotics consisted of 1 gm/liter potassium penicillin G, 0.02 gm/liter streptomycin sulphate, and 0.01 gm/liter terramycin; and were added to all subsequent decalcification medium.

The experimental flasks (50 ml Ehrenmeyer) contained 25 ml Gold's artificial sea water enriched with $^{45}$CaCl$_2$ (500 μCi/liter) and 3.2 ml of the above decalcified matrix slurry. Incubation of samples were made in the following manner:

<table>
<thead>
<tr>
<th>Flask</th>
<th>Temperature</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23°C</td>
<td>Stirred continually</td>
</tr>
<tr>
<td>2</td>
<td>23°C</td>
<td>Medium boiled before addition of slurry. No stirring.</td>
</tr>
<tr>
<td>3</td>
<td>10°C</td>
<td>Stirred continually.</td>
</tr>
<tr>
<td>4</td>
<td>10°C</td>
<td>Medium boiled before addition of slurry. No stirring.</td>
</tr>
</tbody>
</table>
When the "specific activity" (CPM/ml) reached a peak, 5 ml aliquots from each flask were transferred to 1) identical incubating medium or 2) calcium-free Gold's artificial sea water to measure exchange and efflux. Sampling was continued until a trend was observed in calcium uptake or retention. The results of the labelling, before and after transfer, are presented in Figure 92. The 10°C, stirred matrix samples continued to show uptake over the 42 hours incubation while all the other samples reached an equilibrium concentration or decreased in radiocalcium content by 42 hours. The continued uptake by the 10°C stirred sample in a calcium-free medium can be explained by the transfer of calcium in the 5 ml slurry removed from the original incubation flask for transfer. The lack of continued uptake by the stagnant 10°C flasks is probably due to lack of circulation of the calcium ions and matrix.

After incubation for 42 hours, the final flasks were treated in two ways with calcium-free Gold's artificial sea water: 1) The supernatant in the 27°C flasks was completely replaced with Gold's calcium-free sea water after centrifugation to concentrate the matrix. 2) In the 10°C flasks, one-half of the supernatant was replaced with calcium-free Gold's sea water after each centrifugation. One milliliter samples of the matrix were removed after each dilution at intervals of approximately 20 minutes to determine the relative amounts of calcium being removed, or, conversely, the degree of binding of the calcium to the matrix. The results are presented in Figure 93. It can be seen in these figures that the radiocalcium of the 27°C samples exponentially decreases until it
Calcium uptake and retention in decalcified *Porolithon gardineri* matrix. At the maximum radiocalcium uptake, the matrices were transferred to identical medium or to calcium-free medium.
Figure 93

Calcium retention in decalcified *Porolithon gardineri* matrix with supernatant dilution. The 27°C samples had the supernatant completely replaced with calcium-free medium at each dilution. The 10°C samples had the supernatant diluted in half with calcium-free medium at each dilution.
reaches background by the seventh dilution. Decreasing the calcium concentration 50% at each dilution in samples incubated at 10°C resulted in a slower rate of calcium loss. This suggests some interaction with the calcium concentration of the supernatant. The retention of calcium was higher in stagnant samples at both 27°C and 10°C than in the stirred samples. This is probably due to lack of mixing with the diluted supernatant.

**Effects of temperature, oxygen availability, and autoclaving on calcium uptake and retention by decalcified coralline matrix.**

To the coarse *Porolithon gardineri* sand was added an initial antibiotic solution of 5 gm/liter chloromycetin succinate, 17 gm/liter potassium penicillin G and 8 gm/liter streptomycin sulphate. This solutions was diluted to 500 ml with neutral EDTA, and the sand decalcified for 7 weeks at 4°C. After decalcification, a portion of the matrix was autoclaved for 15 minutes. The principal aim was to inactivate any enzymes possibly associated with calcium uptake. A secondary aim was to effect hydrolysis, dissociation or exposure of reactive sites for calcium deposition. All of the experimental flasks were filled with 20 ml of sterile, calcium-free Gold's artificial sea water, 5 ml of matrix slurry, 1.2 uCi/ml $^{45}$CaCl$_2$ (0.6µg/ml), and antibiotics which consisted of 1,000 units/ml of both nystatin and penicillin G. The following experimental treatments were employed for both autoclaved and non-autoclaved matrices:

<table>
<thead>
<tr>
<th>Flask size</th>
<th>Temperature</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 ml</td>
<td>23°C</td>
<td>Swirled frequently, cotton plugged.</td>
</tr>
<tr>
<td>25 ml</td>
<td>23°C</td>
<td>Medium boiled before slurry added, tightly stoppered.</td>
</tr>
<tr>
<td>50 ml</td>
<td>5°C</td>
<td>Swirled frequently, cotton plugged.</td>
</tr>
<tr>
<td>25 ml</td>
<td>5°C</td>
<td>Medium boiled before slurry added, tightly stoppered.</td>
</tr>
</tbody>
</table>
Samples were removed and treated as in the previous experiment. At equilibrium, transfer to non-radioactive medium was also used to estimate rate of exchange as in the previous experiment. The results are presented in Figure 94 and 95. In all of the non-autoclaved matrix samples, calcium appeared to be taken up at a slower rate initially than in the autoclaved matrix. But after 140 hours, the autoclaved matrices had lower relative CPM than the non-autoclaved matrices. As would be expected, samples transferred to calcium-free medium had lower relative CPM than samples transferred to calcium-containing medium. The 23°C samples showed no change in relative CPM after transfer, but there was a small increase in the relative CPM of the 5°C non-autoclaved matrix samples transferred to calcium-free medium.

After centrifugation, half of the supernatant was replaced with calcium-free medium, and centrifugation and replacement repeated 5 times. In Figure 96, the data is presented in a Lineweaver-Burk plot, in which \( \frac{1}{v} \) (where \( v \) = measured velocity) is plotted against \( \frac{1}{(S)} \) (where \( S \) = concentration of the substrate, i.e., calcium). The dissociation constant, \( K_m \), is normally calculated in the equation

\[
\frac{(S)}{V} = \frac{K_m}{V} + \frac{(S)}{V}
\]

(where \( V \) = maximum velocity). The constant, \( K_m \), is calculated from the point where the line intersects the abscissa, the intersection equal to \(-\frac{1}{K_m}\). Hence, it can be observed in Figure 96 that the 23°C decalcified/autoclaved samples have a higher \( K_m \) (matrix dissociation) than the non-autoclaved samples. The 5°C samples have
Calcium uptake and retention by decalcified *Porolithon gardineri* matrices, autoclaved and non-autoclaved, at 23°C. At the maximum radiocalcium uptake, transfers were made to identical medium or to calcium-free medium. The O₂-poor conditions were 25 ml of medium and matrix in a 25 ml flask, tightly stoppered. The stirred flasks were swirled frequently and contained 25 ml of medium and matrix in a 50 ml flask.
Calcium uptake and retention by decalcified *Porolithon gardineri* matrices, autoclaved and non-autoclaved, at 5°C. At the maximum radiocalcium uptake, transfers were made to identical medium or to calcium-free medium. The O₂-poor conditions were 25 ml of medium and matrix in a 25 ml flask, tightly stoppered. The stirred flasks were swirled frequently and contained 25 ml of medium and matrix in a 50 ml flask.
Figure 96

Reciprocal plots of calcium retention by decalcified matrices of Porolithon gardineri, autoclaved and non-autoclaved, at 25°C and 5°C. $1/v = 1/$CPM of sample relative to an initial sample. $1/(S) = 1/$calcium concentration in supernatant.
much the same slope, only the stirred, non-autoclaved sample has a slightly higher (0.15) \( K_m \) (Figure 96).

**Effects of temperature, autoclaving and strontium on calcium uptake and retention by decalcified coralline matrix.** Preparation of the coralline algae for determination of calcium uptake followed the same procedure as for previous experiments, with exception that autoclaving was done for 30 minutes instead of 15 minutes. In brief, the procedure followed was incubation of decalcified *Porolithon gardineri* matrix in artificial sea water containing radiocalcium. After 12 hours, aliquots were taken for dilution of the calcium concentration in the supernatant. Dilution was accomplished by replacing 50\% of the supernatant with calcium-free sea water at intervals of 20 minutes. Samples of 1 ml of the matrix were taken at each dilution for calcium measurement.

The experimental temperatures were set at 5°C, 17°C, 22°C, 29°C, and 36°C. One set of flasks at 22°C contained equal amounts of strontium (SrCl\(_2\)) and radiocalcium (\(^{45}\)CaCl\(_2\)), 0.025 µg/ml of each, to determine if there was competition between strontium and calcium or if calcium was necessary for activation of a physiological process. The 125 ml Erlenmeyer flasks contained 40 ml of sterile calcium-free Gold's artificial sea water, 10 ml of matrix slurry and 0.05 µCi/ml \(^{45}\)CaCl\(_2\) (0.025 µg/ml). No antibiotics were added to the experimental medium, as no transfers beyond 8 hours were anticipated. Radiocalcium uptake was measured using the following experimental conditions:
The table below summarizes the conditions tested:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Matrix</th>
<th>Cation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5°C</td>
<td>1) decalcified only</td>
<td>45CaCl₂</td>
</tr>
<tr>
<td></td>
<td>2) decalcified/autoclaved</td>
<td></td>
</tr>
<tr>
<td>17°C</td>
<td>1) decalcified only</td>
<td>45CaCl₂</td>
</tr>
<tr>
<td></td>
<td>2) decalcified/autoclaved</td>
<td></td>
</tr>
<tr>
<td>22°C</td>
<td>1) decalcified only</td>
<td>45CaCl₂</td>
</tr>
<tr>
<td></td>
<td>2) decalcified/autoclaved</td>
<td></td>
</tr>
<tr>
<td>29°C</td>
<td>1) decalcified only</td>
<td>45CaCl₂</td>
</tr>
<tr>
<td></td>
<td>2) decalcified/autoclaved</td>
<td></td>
</tr>
<tr>
<td>36°C</td>
<td>1) decalcified only</td>
<td>45CaCl₂</td>
</tr>
<tr>
<td></td>
<td>2) decalcified/autoclaved</td>
<td>SrCl₂</td>
</tr>
<tr>
<td>22°C</td>
<td>1) decalcified only</td>
<td>45CaCl₂</td>
</tr>
<tr>
<td></td>
<td>2) decalcified/autoclaved</td>
<td></td>
</tr>
</tbody>
</table>

The relative radiocalcium uptake for these conditions is shown in Figure 97. It can be seen that the rate of 45Ca uptake and total calcium uptake in the 17-36°C temperature range was slower for the autoclaved matrix than that for only decalcified matrix. The rate of calcium uptake is faster and total calcium uptake is greater for autoclaved matrix than for non-autoclaved matrix incubated at 5°C.

In the incubation medium containing equal amounts of radiocalcium and strontium, the calcium uptake by decalcified-only matrix was approximately 0.5 that for the uptake by identical matrix under the same conditions without strontium. This experiment does not indicate if the strontium was incorporated with calcium or if only calcium was taken up. In contrast, the autoclaved matrix attained its highest calcium uptake in the calcium/strontium medium (Figure 97).

After 8 hours, 6 ml of the resuspended matrix plus incubating medium were removed for supernatant dilution 1:1 with Gold's artificial sea water, calcium-free, as in the preceding experiment. The reciprocal plots (Lineweaver-Burk) with the linear regression
Figure 97

Calcium uptake by decalcified *Porolithon gardineri* matrices, autoclaved and non-autoclaved, at various temperatures and with strontium added in equal amount to calcium in one set of flasks, 22°C + Sr.
included are presented in Figure 98. Loss of calcium from the matrix has essentially the same $K_m$ for autoclaved and/or decalcified samples at 5°C, 36°C and equal parts of calcium and strontium at 22°C. Lower values for $K_m$ are noted for decalcified-only matrix at 17°C, 22°C and 29°C.

The uptake of calcium by the matrices in relation to the calcium concentration in the supernatant was determined for samples after 6 hours and 192 hours (Table 13). The decalcified-only matrix was added to the same $^{45}$CaCl$_2$-enriched medium at a lower concentration than the decalcified/autoclaved matrix. Adjustment for this difference was made by comparison of the initial CPM for the autoclaved and/or decalcified samples from each temperature. The final adjustment factor was determined as follows:

$$\frac{\text{CPM decalcified/autoclaved matrix, initial}}{\text{CPM decalcified matrix, initial}} = \text{Adjustment factor}$$

and the relative calcium uptake equation:

$$\frac{\text{CPM decalcified matrix}}{\text{CPM supernatant}} \times \text{Adjustment factor}^a = \text{Relative calcium uptake matrix/supernatant.}$$

$a$ Adjustment not used for decalcified/autoclaved matrix.

The results show decalcified/autoclaved matrices at approximately equilibrium with the supernatant after 192 hours. The decalcified-only matrices vary in calcium uptake from equilibrium with the supernatant at 5°C to 2 times the supernatant calcium concentration at 36°C. Because cold, neutral decalcification of the matrix allows calcium uptake against a concentration gradient, a physiological factor is suggested as well as a physical precipitation occurring.
Figure 98

Reciprocal plots of calcium retention by decalcified matrices of *Porolithon gardineri*, autoclaved and non-autoclaved, at various temperatures and with strontium added in equal amount to calcium in one set of flasks, 22°C + Sr.

$1/v = 1/\text{CPM of sample relative to an initial sample.}$

$1/(S) = 1/$calcium concentration in supernatant
Table 13

Effect of Calcium Concentration on the Calcium Uptake by Decalcified or Decalcified and Autoclaved Matrix as a Function of Temperature and Time of Incubation.

<table>
<thead>
<tr>
<th>Matrix Sample</th>
<th>Relative CPM of Matrix*/Supernatant**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 hours</td>
</tr>
<tr>
<td>5°C Decalcified</td>
<td>0.647</td>
</tr>
<tr>
<td>Decalcified/Autoclaved</td>
<td>0.707</td>
</tr>
<tr>
<td>17°C Decalcified</td>
<td>1.330</td>
</tr>
<tr>
<td>Decalcified/Autoclaved</td>
<td>0.752</td>
</tr>
<tr>
<td>22°C Decalcified</td>
<td>0.985</td>
</tr>
<tr>
<td>Decalcified/Autoclaved</td>
<td>0.838</td>
</tr>
<tr>
<td>29°C Decalcified</td>
<td>1.180</td>
</tr>
<tr>
<td>Decalcified/Autoclaved</td>
<td>0.754</td>
</tr>
<tr>
<td>36°C Decalcified</td>
<td>1.610</td>
</tr>
<tr>
<td>Decalcified/Autoclaved</td>
<td>0.883</td>
</tr>
<tr>
<td>22°C equal parts of strontium and calcium</td>
<td></td>
</tr>
<tr>
<td>Decalcified</td>
<td>0.612</td>
</tr>
<tr>
<td>Decalcified/Autoclaved</td>
<td>0.655</td>
</tr>
</tbody>
</table>

* The decalcified matrix was adjusted for difference in amount from the autoclaved decalcified matrix as explained in text.

** The CPM of the supernatant were taken at 8 hours for both matrix sample times.
That the difference in calcium uptake was related to bacterial contamination was considered as a possible explanation for these results. Sterile medium was used in the experiments for the incubation with $^{45}$CaCl$_2$-enriched calcium-free Gold's artificial sea water. However, no attempt was made to employ sterile technique in the removal of aliquots nor in the addition of the non-autoclaved matrix (previously treated with a strong antibiotic solution). The validity of the results of calcium uptake by the matrices was checked by making streaks with a sterile loop from the 17°C flasks one month after the beginning of the experiment. Two types of agar culture plates, 1) ZeBell's medium (2216E) (ZeBell, 1946) and 2) Trypticase Soy Agar (Baltimore Biological Laboratory, Baltimore) were used for testing bacterial contamination of the solutions. After 24 hours at room temperature (23°C), the autoclaved matrix showed slightly more bacterial growth than the non-autoclaved matrix on both types of agar culture plates. These results give indication that bacteria can be discounted as the causative factor for the higher calcium uptake by non-autoclaved matrix.

**Effect of ATP on calcium uptake and retention by decalcified coralline matrix.** To test the possibility of an energy requiring system involved in calcium uptake by the decalcified matrix, ATP (adenosine triphosphate) was added to the experimental flasks. The 50 ml flasks contained 20 ml of calcium-free Gold's artificial sea water, 5 ml of matrix slurry, 1.2 µCi/ml $^{45}$CaCl$_2$ and 0.25% ATP. Both decalcified and decalcified/autoclaved matrix samples were used. The flasks were kept at 22°C with samples taken at intervals
for 26 hours. Dissociation of the calcium from the matrix was determined by the dilution technique described for Experiments 3 and C. Controls consisted of identical flasks without ATP added. There is little difference between the decalcified matrix with ATP added and the autoclaved/decalcified samples, control and added ATP. The results for calcium dissociation from the matrices with and without ATP added showed little difference. The decalcified matrix had been kept at 10°C in calcium-free Gold's artificial sea water for over a month, and the results suggest that any physiological factor taking part in calcium uptake by decalcified matrix previously was not involved in this experiment.

**Calcium uptake by polysaccharides**

Two polysaccharides, xylan and cellulose, were tested for calcium uptake. Xylan powder (obtained from Dr. C. C. Tu, Hawaiian Sugar Planters Association, Honolulu) and cellulose powder (Whatman CF 11, fibrous cellulose powder) were chosen because of previous investigations naming them as constituents of the coralline cell wall (page 161). Cellulose was also used because of its birefringence and known highly ordered structure.

The cellulose powder was suspended about 10 hours in calcium-free Gold's artificial sea water. The xylan powder was suspended in acetate buffer, pH 6.1, in order to prevent solubilization of the xylan. Aliquots from the cellulose and xylan suspensions were autoclaved at 20 p.s.i. for 30 minutes at 250°C. After cooling overnight, the xylan powder remained as a fine suspension and centrifugation was necessary, resulting in approximately 2/3 recovery of
the original amount. Added to 50 ml Erlenmeyer flasks were 5 ml of the polysaccharide slurry, 20 ml of calcium-free Gold's artificial sea water and 1.2 µCi/ml of $^{45}$CaCl$_2$. Sampling was done as with the *Porolithon gardineri* matrix (see Materials and Methods) for 6 hours with no subsequent transfers. The results are given in Figure 99.

The CPM for cellulose powder are essentially equal to a control millipore filter (60 ± 10), showing no uptake nor differences between autoclaved and/or suspended cellulose powder. The xylan powder showed a small uptake of radiocalcium, with the autoclaved portion demonstrating a higher label. The autoclaved powder has significantly higher CPM if it is considered that approximately 0.3 less autoclaved xylan is present than in the other sample. However, there is no further calcium uptake at 6 hours after the initial amount at approximately 5-10 minutes.

**X-ray diffraction of Porolithon gardineri matrix.**

When filtering decalcified matrix after exposure to calcium, a distinct if subjective difference was noted between autoclaved and non-autoclaved samples. This difference became apparent after approximately 4 hours in the calcium-enriched Gold's sea water, and, along with the subjective observations, the autoclaved matrix took longer to filter dry on the millipore filter. X-ray diffraction was employed to determine structural dissimilarities, and the results are presented in Figure 100.

Matrices from Experiment C, 17°C, were filtered and dried at room temperature on Whatman #1 filter paper. The dry powder was packed in X-ray diffraction powder holders and scanned from 10° to 60° 2θ. Autoclaved and/or decalcified matrix which was kept at 4°C
Figure 99

Calcium uptake by xylan and cellulose, autoclaved and non-autoclaved. Shaded area indicates background CPM.
XYLAN, AUTOCLAVED

XYLAN, NO TREATMENT

CELLULOSE, AUTOCLAVED

CELLULOSE, NO TREATMENT

TIME (HOURS)

CPM
Figure 100

X-ray diffraction patterns of decalcified *Porolithon gardineri* matrices, autoclaved and non-autoclaved, before and after incubation in a calcium-containing medium. Arrows indicate new peaks after incubation.
without incubation in calcium was diffracted in the same way. Peaks of the four samples were compared before and after calcium incubation.

In Figure 100, arrows mark peaks occurring after calcium incubation. The autoclaved and/or decalcified matrices have the same X-ray diffraction pattern before incubation in calcium-enriched Gold's artificial sea water. After incubation, the autoclaved matrix shows new peaks scattered through the diffraction pattern. The decalcified-only matrix has only three obvious new peaks after calcium incubation, occurring at 17.0, 21.2, and 31.5° 2θ. Several of the peaks marked by arrows in the autoclaved and decalcified matrix compare to aragonite peaks (see Figure 35). However, interpretation of the peaks, and d-spacing, was deemed beyond the scope of the present investigation. The X-ray diffraction peaks, their location and occurrence in the different matrices are listed in Table 14.

Discussion

Calcium incorporation into coralline thalli was investigated to obtain data as to movement and eventual crystal formation. Initially, experiments were conducted with non-living, decalcified tissue. The results of radiocalcium uptake by geological thin sections were monitored for information on whether uptake occurred, the gross rate and possible sites of concentration. The decalcified sections, showing increased calcium with increased time incubated in a medium containing calcium (Figure 83) provided enough data to warrant more carefully controlled and precise experiments.

Coralline thalli grown on cover slips, both calcified and decalcified, were incubated in a medium with radiocalcium and prepared
Table 14
X-ray Diffraction Peaks of Autoclaved and/or Decalcified Matrix With and Without Calcium Incubation

<table>
<thead>
<tr>
<th>2θ</th>
<th>d spacing</th>
<th>Matrix</th>
<th>Decal</th>
<th>Decal +Ca</th>
<th>Auto</th>
<th>Auto +Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.47</td>
<td>7.708</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.75</td>
<td>6.937</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>14.9</td>
<td>5.941</td>
<td></td>
<td></td>
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<tr>
<td>17.0</td>
<td>5.211</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>21.2</td>
<td>4.817</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>22.7</td>
<td>3.914</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>23.5</td>
<td>3.782</td>
<td>y</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>26.1</td>
<td>3.411</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29.9</td>
<td>2.986</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>31.7</td>
<td>2.820</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
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</tr>
<tr>
<td>32.1</td>
<td>2.786</td>
<td>x</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>33.1</td>
<td>2.704</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>34.3</td>
<td>2.612</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>36.5</td>
<td>2.460</td>
<td>x</td>
<td>x</td>
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</tr>
<tr>
<td>37.8</td>
<td>2.378</td>
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<td></td>
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<tr>
<td>40.1</td>
<td>2.247</td>
<td>x</td>
<td>x</td>
<td>x</td>
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</tr>
<tr>
<td>43.9</td>
<td>2.061</td>
<td>x</td>
<td>x</td>
<td>x</td>
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<tr>
<td>45.4</td>
<td>1.996</td>
<td>x</td>
<td></td>
<td></td>
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<tr>
<td>48.5</td>
<td>1.875</td>
<td>x</td>
<td>x</td>
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<tr>
<td>49.3</td>
<td>1.847</td>
<td>x</td>
<td>x</td>
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</tr>
<tr>
<td>54.3</td>
<td>1.674</td>
<td></td>
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</tr>
<tr>
<td>58.4</td>
<td>1.579</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
</tbody>
</table>
for radioautography to determine calcium uptake and pathways. The results of uptake by decalcified thalli (Table 11) show what appears to be an immediate adsorption of calcium in the 22°C thalli (decalcified at 22°C and air dried), with maximum label at 6 hours scattered indiscriminately over the entire thalli. In contrast, the thalli decalcified at 4°C and immediately transferred to the radiocalcium incubation medium showed a progressive uptake of calcium over the 24 hours sampled. The moderate label occurring in the thalli centers at 1 hour and remaining constant over 24 hours suggests a replacement of the calcium removed by EDTA from calcified cell walls and cells. The increasing label in the cover cells and algal periphery over 24 hours implies an active incorporation of calcium in those areas. This supposition would be in agreement with high metabolic activity in the cover cells and thallus periphery suggested by previous experiments with radiosulphur uptake. Cover cells seem to have a more active and important role in coralline metabolism than is inferred from the literature (Fritsch, 1959). The closely packed lamellae, dense cytoplasm and numerous mitochondria (Figure 31) indicate an area of potentially high activity.

Enzymes may be found associated with disulphide groups. These enzymes are activated by substances which reduce the disulphide to a sulphydryl group (Fruton and Simmonds, 1959). In many plants, glutathione (with SH groups) is an important activator, which also serves as a regulator of enzyme activity. Sulphated polysaccharides were indicated in cover cells by the alcian blue staining (Figure 67), and disulphide bonds were demonstrated with ferric ferricyanide.
stain (Figure 71). The role of the enzymes may be connected with formation of an organic matrix which contains nucleation sites or with the activation of sites for calcium carbonate. Lewin (1962) found sulphydryl groups connected with silicon uptake in diatoms, disclosing possible correlations in calcium and silicon deposition.

Calcite nuclei formed on coated electron microscope grids inserted between the mantle and shell of an oyster (Wilbur and Watabe, 1967). The nuclei, identified by diffraction as calcium carbonate, ranged in size from 75 - 150Å and occurred in the roughly round form found on the coralline cell wall fibres. The crystal nuclei, or condensations, found on the decalcified coralline matrix (Figures 84, 85, and 86) probably are not calcium carbonate. Extensive decalcification was done with EDTA, and the nuclei may be the result of new precipitation on the fibres or phosphate or carbonate compounds which serve as nucleation sites.

Fibre condensations are apparent in Figure 86, appearing in various sizes and shapes. Mueller and Szent-Gyorgyi (1957) found muscle fibres contracted as much as 0.2 times the original length if put in water. To avoid this contraction and separation of fibrils, wet freeze-drying using cold acetone and ethyl chloride was successfully employed. Scott and Nylen (1959), finding widely separated microfibrils in tendon, tried the wet freeze-drying technique and showed sheets of fibrils. Apparently, the same effects of aggregation and separation occur in the coralline cell wall fibres with OsO₄ post-fixation (Figure 86). The cell wall fibrils occur randomly, yet the suggestion of broad sheets of fine fibrils occurs in Figure 84 and 85.
Double fibrils, 100-150Å wide, are seen abutting on or alongside crystals in Figures 88 and 90, sections of calcified coralline alga. The double fibrils of 150Å may be the result of fibres held together by crystal nucleation sites forming a stronger bond than that responsible for intrafibril arrangement. In Figure 91 at the solid arrow, fine extensions can be seen reaching from one fibril in a 300Å couplet to the other fibril. It is feasible that growth of the crystals produces wider spacings, from the 150Å to the 300Å. Many of the 200Å (150Å?) double fibrils can be seen in the shadow cast section (Figure 90) as well as numerous intercrystal bridges with no fibrillar structure visible. The double fibrils have a fine periodicity of approximately 80Å, also found on the edges of some crystals (Figure 89). It appears that the periodic fibrils in the cell wall loosen as crystals are formed and move into the intercellular area. This causes the rows of crystals perpendicular to the cell wall that can be seen to occur some distance away from the cell (Figure 88). A tight coherence between the matrix and crystal would support the existence of nucleation sites in the matrix and the fibril dimension changes. A summary of cell wall fibril sizes and periodicities is given in Table 15 below.

Table 15

<table>
<thead>
<tr>
<th>Description</th>
<th>Width</th>
<th>Periodicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>double fibrils, Figure 88</td>
<td>100-150Å</td>
<td>50-80Å</td>
</tr>
<tr>
<td>double fibrils, Figure 88, 89</td>
<td>200Å</td>
<td>50-80Å</td>
</tr>
<tr>
<td>double fibrils, Figure 91</td>
<td>250-300Å</td>
<td>300Å</td>
</tr>
<tr>
<td>single fibril, Figure 85</td>
<td></td>
<td>300Å</td>
</tr>
<tr>
<td>single fibril, Figure 84, 86</td>
<td></td>
<td>700Å</td>
</tr>
<tr>
<td>initial crystal seeding, Fig. 56</td>
<td></td>
<td>150Å</td>
</tr>
</tbody>
</table>
From the electron micrographs presented in Chapter III on crystal development and in this chapter, it is obvious that fibre periodicity is present. The disparity in periodicities (Table 15) is difficult to relate in the different electron micrographs. Until further sections in known planes are available for study, only a conjecture is feasible to explain the data. It is conceivable that the 80Å periodicity occurs on fibrils in one direction and the 300Å periodicity is either a result of fibre spacing or larger periodicity on other fibrils. The visualization of the "sheaths" occurred only in one series of sections, suggesting that the sectioning angle or location was responsible for elucidating the structures. A diagram of a proposed arrangement is presented in Figure 101.

Crystallization was first seen at 150Å spacings at high magnification (Figure 56), with the sectioning plane perpendicular to the direction of growth (Figure 101B). The 150Å crystal nucleation may be the result of seeding on both sides of the 50-80Å periodic fibres. Or the cell wall fibres may slacken and the periodicity decrease as the fibres converge at the pit connection (Figure 101A). The 50-80Å periodicity was found where the sectioning plane was unknown.

Watabe and Wilbur (1961) found a very fine, oriented pattern in the conchiolin membrane of the oyster, on which smaller crystals than those found in zones on either side of the membrane were formed. As they grew towards the shell center, the crystals increased in size and formed multilayers. Thus, crystals associated with an organic matrix and growing in size have been found. In an organic system, only a few large molecules may be necessary to form an initiating nucleus (Walton, 1965). The cell wall fibrils in the corallines
Figure 101

Diagrams of corallines with proposed structure to correspond to described periodicities. A coralline cell with postulated fibre orientation and periodicity is shown in A. A coralline alga on a cover slip, showing planes of sectioning producing different periodicities, is presented in B.
CRYSTAL NUCLEI

300Å PERIODICITY

PIT CONNECTION

80Å (OR 150Å) PERIODICITY

150Å

80Å

"SHEATHS"

300Å

80Å
appear to act as nucleation sites and, by disengaging from the underlying layer as crystals form, provide a continuing substrate until the intercellular area is filled with crystals.

Decalcification of Porolithon gardineri branches provided an organic matrix with which to investigate physiological processes associated with calcium uptake. The results of all the experiments are best considered together, since data from earlier experiments were used as a basis for further research. Decalcified matrices took up radiocalcium faster initially at higher temperatures (Figures 92 and 97). After being transferred, the lower temperature samples incorporated calcium at a faster rate for long periods of time (34 to 140 hours) than the warmer matrices. The uptake of one experiment demonstrating the trend is summarized in Table 16.

Table 16

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Relative to initial CPM</th>
<th>Relative to initial transfer CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>10°C stagnant</td>
<td>2.3</td>
<td>+Ca 9.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Ca 7.0</td>
</tr>
<tr>
<td>10°C stirred</td>
<td>3.3</td>
<td>+Ca 13.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Ca 9.6</td>
</tr>
<tr>
<td>27°C stagnant</td>
<td>17.0</td>
<td>+Ca 7.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Ca 3.3</td>
</tr>
<tr>
<td>27°C stirred</td>
<td>16.4</td>
<td>+Ca 2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Ca 1.8</td>
</tr>
</tbody>
</table>

* Maximum sampling time
Not only did the moderate temperatures (10-17°C) take up calcium over a longer period, but they retained more of their original count after supernatant dilutions (Figures 96 and 98). At 5°C and 36°C, there was less difference between the two groups (Figure 98). The coralline data strongly parallel the temperature optima in coccolithophorids (Watabe and Walsby, 1966). They found highest growth rates at 18-24°C, with 18°C optimum. The dissociation of calcium from the matrix by supernatant diluting tended towards a higher $K_m$ for the decalcified-only samples, except at 5°C. This suggests stronger bonds than were formed in the autoclaved matrix, presumably necessitating energy expenditure by the matrix. Active processes in non-autoclaved decalcified matrices (active) and thalli were also suggested by labelling thalli with calcium, incorporation of calcium against a concentration gradient and longer periods of calcium uptake by the decalcified matrix, and temperature optima.

With later evidence obtained from diffraction of the matrix indicating incomplete decalcification, there immediately occurred the question of whether calcium uptake was merely an exchange reaction or replacement of the EDTA-removed calcium. However, autoclaving breaks up the matrix, exposing more surface area on which the physical processes may occur. Since the non-autoclaved matrix already showed a higher uptake of calcium, the difference would only be made greater if adjustment for the physical process was made.

The slower uptake at low (5°C) temperature and a temperature optimum at 17°C as well as data that plotted well on the Lineweaver-Burk reciprocal graphs (Figure 98) suggest a physiological process.
involved in calcium uptake. It appears that at 5°C, physiological processes involved with calcium uptake are curtailed, resulting in uptake by physical means. The higher uptake at 36°C by the non-autoclaved matrix (active) is contradictory if enzymes are postulated, as they would be expected to be inactivated at that temperature. Calcium carbonate goes into solution more readily at low temperatures than at high ones, however, and physical precipitation on the fibres or other crystals may account for the higher uptake values and lower binding for the non-autoclaved matrix. The least loss of calcium from a matrix (strongest binding) occurred at 29°C with non-autoclaved matrix. Uptake of calcium was greatest at 17°C with identical matrices, seemingly associated with optimal growth temperature (Chapter II and Figure 15). The disparate temperatures for calcium uptake and strongest binding are not surprising, as there is no reason to assume that calcium incorporation is associated with optimal growth conditions. The uptake by autoclaved matrix and geological thin sections proves a physical uptake as well.

To obtain more insight into the physiological reactions, a medium containing equal amounts of calcium and strontium was used for matrix incubation. Both ions were present in low concentration (0.025 µg/liter) as compared to 0.03 g/liter calcium in Gold's artificial sea water. The decalcified matrix showed approximately 0.5 times the calcium incorporated under identical conditions without strontium (Figure 97). This result may be interpreted in two ways, 1) there is no preference for either cation and equal uptake occurs, or 2) strontium inhibits calcium uptake, possibly by
enzyme interference. The experiment was not designed to measure strontium uptake, only radiocalcium, so there is no way to tell if equal amounts of strontium and calcium or only half the normal calcium is incorporated. Because the $K_m$ of the decalcified matrix approximates the $K_m$ of the autoclaved matrix so closely, the second explanation seems more plausible. In contrast to the low calcium uptake by decalcified-only matrix, the highest calcium amount and rate by the autoclaved matrix was reached with strontium present. This suggests calcium uptake by autoclaved matrix as an inactive replacement reaction, dependent on the cation concentration of the supernatant.

Equilibrium exchange in dead tissue was discussed previously in relation to intracellular crystals (page 155). Uptake of calcium by decalcified-only matrices reached 2 times the concentration in the supernatant (Table 13) while autoclaved matrices did not exceed equilibrium with the supernatant, suggesting active uptake against a concentration gradient by coralline algae.

A matrix on which crystals can precipitate is an essential part of biological calcification. Configuration of the molecules is important to mineralization (Glimcher, 1960), and polysaccharides such as cellulose show birefringence and a highly ordered structure. Xylan and cellulose powders were tested for possible calcium attachment. Xylose, indicated as a possible cell wall constituent by the index of refraction of coralline matrix (Table 10), incorporated a small but significant label (Figure 99). Cellulose stayed at background throughout the experiment. Comparisons of calcium uptake by cellulose, xylose and the cell wall matrix are given in Table 17.
Table 17
Calcium Uptake by Coralline Matrix and Polysaccharides

<table>
<thead>
<tr>
<th>Sample</th>
<th>% of comparable matrix label</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decalcified matrix</td>
<td>100</td>
</tr>
<tr>
<td>Autoclaved matrix</td>
<td>100</td>
</tr>
<tr>
<td>Xylan</td>
<td>9.6</td>
</tr>
<tr>
<td>Autoclaved xylan</td>
<td>12.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>1.3</td>
</tr>
<tr>
<td>Autoclaved cellulose</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Autoclaved and/or decalcified matrices were diffracted with X-rays to ascertain variation in crystal structure between the two preparations of matrix after incubation in a calcium-containing medium. Before incubation, the matrices gave essentially identical diffraction patterns, indicating incomplete decalcification by the high calcite peaks. After incubation, new peaks occurred. The numerous peaks in the autoclaved matrix suggest less regulation by the matrix, probably due to the exposure of more nucleation sites and less physiological control. The decalcified-only matrix had only three obviously new peaks after incubation. Agreement of the three peaks with the d-spacings of known inorganic crystals (ASTM, Index (Inorganic) to the Powder Diffraction File 1966) was difficult to obtain. Possible crystals are listed in Table 18, chosen because one or more of the d-spacings were the same as those from the diffraction patterns.
Table 18

D-spacings of Diffraction Pattern of Incubated, Decalcified Coralline and Some Possible Crystal Components

<table>
<thead>
<tr>
<th>Crystal</th>
<th>Significant d-spacings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubated matrix, new peaks</td>
<td></td>
</tr>
<tr>
<td>Calcium phosphate Ca$_2$P$<em>6$O$</em>{17}$</td>
<td>5.16  4.60  4.02</td>
</tr>
<tr>
<td>Calcite (calcium carbonate) (Ca, Mn, C)</td>
<td>3.04  2.29  2.10</td>
</tr>
<tr>
<td>Vaterite</td>
<td>3.58  3.30  2.73</td>
</tr>
<tr>
<td>Aragonite</td>
<td>3.40  3.27  1.98</td>
</tr>
<tr>
<td>Calcium carbonate-sodium</td>
<td></td>
</tr>
<tr>
<td>Na$_2$Ca(CO$_3$)$_2$·2H$_2$O</td>
<td>5.10  2.65  2.50</td>
</tr>
<tr>
<td>Calcium chloride oxalate hydrate</td>
<td></td>
</tr>
<tr>
<td>C$_2$Ca$_2$Cl$_2$O$_4$·2H$_2$O</td>
<td>5.24  2.32  2.95</td>
</tr>
<tr>
<td>Calcium thiosulphate hydrate</td>
<td></td>
</tr>
<tr>
<td>CaS$_2$O$_2$·6H$_2$O</td>
<td>5.33  2.89  4.24</td>
</tr>
</tbody>
</table>

The distinguishing of three significant peaks for a specific crystal is not always possible, because other peaks may overlap and cover lesser ones. Thus, the peak at 5.21 in the decalcified matrix was chosen for determination of the unknown crystal because it was relatively high and isolated.

Calcium oxalate crystals are found in intracellular locations in plants (Arnott and Pautard, 1970), supporting the presence of calcium chloride oxalate in the incubated coralline matrix. No indication of this crystal was found before decalcification and subsequent incubation, and the crystal formation may be due to physiological conditions in the incubating medium.
Carbonate substitutes for phosphate in calcium phosphate (LeGeros, Trautz, LeGeros, Klein and Shirra, 1967). The presence of carbonate causes the formation of weak bonds, and crystal growth is then in the general direction of weak bonds instead of the formation of strong bonds. The absence of calcium phosphate peaks before incubation may be the result of completed carbonate substitution in the calcified coralline, or physiological conditions may cause an anomalous crystal to be formed.

With the data available, it would be presumptive to state more than X-ray diffraction patterns are changed during post-decalcification incubation in radiocalcium-enriched Gold's artificial sea water and the possible crystals. However, the pattern differences between autoclaved and/or decalcified matrices incubated in identical medium suggest structural and/or physiological influence on crystal type.

From the data on the coralline organic matrix presented in this chapter, it can be seen that there exists a potentially calcifiable substrate. The cell wall fibrils have the periodicity associated with mineralizing matrices, calcium is taken up and moved discriminately within live coralline thalli, temperature affects calcium uptake as does autoclaving the matrix, and crystal structure varies with calcium deposition on autoclaved and/or decalcified matrix.

The actual physiological processes connected with mineralization can only be postulated from the data. Enzyme activity is supported by the response in calcium uptake to variations in temperature, the effect of strontium inhibiting calcium uptake, and enzyme-
sulphydryl association. Carbonic anhydrase or a phosphatase may be involved, but the $K_m$ suggests a single enzyme system in the autoclaved matrices (essentially the same $K_m$ at all temperatures) and a more complicated enzyme system in the non-autoclaved (active) matrices ($K_m$ affected by different temperatures). The oriented crystals at the cell wall indicate a process occurring outside the cell proper. It would appear that intracellular mineralization occurs only after degeneration of the cell affects the plasmalemma and renders the membrane permeable to the calcium cations. Because other highly ordered polysaccharides (cellulose and xylose) did not incorporate calcium to the extent that the coralline matrix did, structure alone would not seem responsible for the high calcium uptake.
SUMMARY

1. The growth rate of coralline algae is adversely affected by temperatures above 24°C and below 10°C, with optimal growth at 17°C. Other factors influencing coralline growth and settling are strong light, wave surge, and light colored substrates, all of which decrease thallus number.

2. The morphology of the coralline algae is described from light and electron microscopy.

3. Histochemical tests on coralline thalli demonstrate the presence of sulphated polysaccharides, protein, lipid and disulphide bonds. No cellulose nor pectin were found. Tests for carbonic anhydrase and acid phosphatase were positive. Metachromasia occurs in a submarginal band of cells which also reacts more strongly with the stains employed. Methylene blue staining at low pH demonstrates strong basophilic qualities in the tissue.

4. Birefringence occurs in the decalcified as well as in the calcified coralline thallus. Calcite produces intrinsic birefringence, but decalcified thalli have an extinction point at 1.518, demonstrating form birefringence in the matrix fibres.

5. Radiocalcium and radiosulphur are concentrated in the cover cells immediately and in the peripheral cells after 12-24 hours. Rate of calcium and sulphur uptake is faster initially in continuous light than under normal conditions. Thalli kept in continuous dark show little uptake and appear to degenerate.

6. The crystal deposited in coralline algae is identified as the calcite polymorph of calcium carbonate by X-ray diffraction and fluorescence. Progressive mineralization occurs in Porolithon
Porolithon gardineri from the center and base of a branch upwards and outwards.

7. Crystal orientation was determined from electron micrographs and diffraction. Initial precipitation is seen at 150Å intervals at the inner edge of the cell wall. The crystals appear to move away from the cell, coalescing into larger crystals. Shadowed sections of coralline algae revealed rounded edges on the tabular crystals of calcite. Crystals overlap others further away from the cell, suggesting outward movement.

8. The organic matrix of the coralline algae consists of sheets of fine, randomly oriented fibrils with a deep staining layer (Pd/MnO₄ and Pb citrate) near the plasmalemma. Periodicities can be seen along the fibres, ranging from 30-700Å. A close matrix-crystal interaction is suggested by double fibrils (150Å) with 30Å periodicity abutting on some crystals. Similar periodicities of 30Å are occasionally seen along the edges of crystals away from the immediate cell edge. An extensive organic matrix network is seen connecting crystals and is left where crystals have fallen out of the sections.

9. In vitro incubation of Porolithon gardineri matrix indicates calcium uptake and retention is influenced by temperature, ion concentration, and autoclaving of the matrix. Xylan and cellulose show little calcium uptake.

10. It is concluded from the above summarized results that the coralline algae present a biological system capable of influencing calcification processes. A potentially calcifiable matrix is demonstrated by development of metachromasia before mineraliza-
tion occurs, strong basophilic properties in the tissue, fibre periodicity, and calcium incorporation. Matrix-crystal interaction is manifested by crystal orientation and location within an extensive organic matrix network. Physiological processes are suggested by greater calcium uptake and stronger binding by cold decalcified matrix than by autoclaved matrix, slower uptake and weaker binding of calcium at temperatures below 10°C and above 29°C, and differences in X-ray diffraction patterns between decalcified, autoclaved matrix and cold decalcified matrix after incubation in a calcium-containing medium.
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